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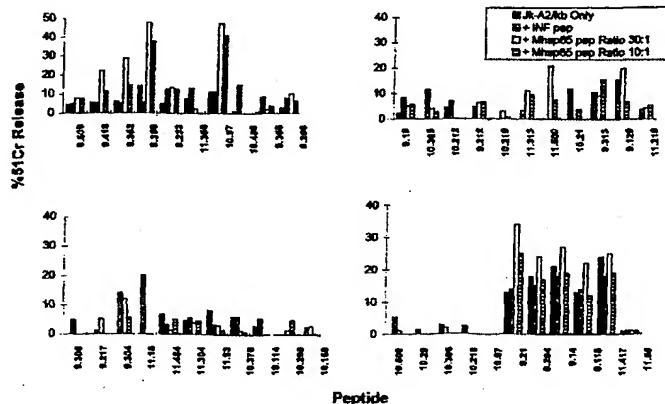
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(54) Title: INDUCTION OF CYTOTOXIC T LYMPHOCYTE RESPONSE BY HLA CLASS IA RESTRICTED EPITOPES OF MYCOBACTERIAL HEAT SHOCK PROTEIN 65



**INDUCTION OF CYTOTOXIC T LYMPHOCYTE RESPONSE BY
HLA CLASS IA RESTRICTED EPITOPES OF
MYCOBACTERIAL HEAT SHOCK PROTEIN 65**

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FIELD OF THE INVENTION

The present invention relates generally to the induction of cytotoxic T lymphocyte (CTL) responses to polypeptides containing epitopes derived from the Mycobacterial heat shock protein 65 as well as to uses of such polypeptides to protect against bacterial and parasitic infections, such as tuberculosis.

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BACKGROUND OF THE INVENTION

Mycobacterium tuberculosis (MTB) is the leading cause of death from a single infectious pathogen, accounting for 10 million new cases and three million deaths annually (1). An attenuated strain of Mycobacterium bovis, Bacille Calmette- Guérin (BCG), is the only available vaccine today. The efficacy of this widely administered vaccine is a subject of controversy, varying from as high as 80% to as little as zero (2). As the incidence of tuberculosis is increasing worldwide, the development of successful new vaccines to replace BCG is urgently needed. A better understanding of the immunological mechanisms involved in the protection from MTB is expected to help in designing better prophylactic as well as therapeutic vaccines (3-7).

It is well established that protective immunity to TB depends on both MHC class II restricted CD4 T helper cells secreting type-1

cytokines, as well as MHC class I restricted CD8 cells (3, 8-15). The antigens which are recognized by CD4⁺ T cells have been in part characterized, as exemplified by the mapping of multiple HLA-DR and – DQ presented epitopes on the mycobacterial hsp65 (Mhsp 65) (16-18).

5 Other examples of T-cell stimulating antigens from MTB are the non-peptidic phospho-ligand presented to gamma- delta TCR⁺ cells by as yet unidentified cell surface molecules, as well as several non-classical epitopes consisting of mycobacterial lipoglycan moieties recognized by double negative $\alpha\beta$ TCR⁺ and a subset of CD8⁺ cells in the context of

10 CD1 molecules (19-22). In contrast, however, the target antigens recognized by classical MHC class I restricted CD8⁺ T cells which presumably represents a major subset of the CD8 compartment, have remained largely unknown (13, 22-25). CD8⁺ T cells may be the most efficient lymphocyte subset in mediating protection from and resistance to

15 tuberculosis (26-31), as concluded from both animal models, such as the β 2 microglobulin (β 2m) knockout mouse (26, 32), antibody depletion and passive transfer experiments of T-cell subsets (28, 33-36), as well as from studies done on tuberculosis patients and healthy human subjects (37-40). The mechanism of MTB facilitated MHC class I presentation was

20 recently shown to be dependent upon the transporter associated with antigen processing (TAP) (41). It is also noteworthy that infected parenchyma cells of the lung are amenable to immuno-surveillance only via classical MHC-I restricted CD8 T-cells because they do not express MHC-II or non classical MHC-I antigen presenting molecules (42, 43). This

25 might explain why the local immune response in the lung is especially dependent on the CD8⁺ T cells (13, 44). Indeed, mycobacteria protein specific and HLA class I restricted killing was recently demonstrated using alveolar lymphocytes (37). Tascon et al have recently reported that immunization with the *M. leprae* hsp65 expressing plasmid could lead to a

30 comparable protection against tuberculosis as that obtained with BCG (45). This protection was correlated with high levels of cytotoxicity and

IFN- γ release and was later reported to be as sustained as that achieved by BCG (46). Also, adoptively transferred protection with hsp 65-reactive T cell clones was reported to be particularly associated with cytotoxicity mediated by CD8⁺ T cells (27, 30). In another study, the CD8⁺ subset of CTLs was the only population of T cells capable of decreasing bacterial growth in infected macrophages in vitro (13). Therefore, efforts to define the antigens as well as the immunodominant epitopes recognized by CD8⁺ T cells are important for the development of vaccines against TB and other intracellular pathogens.

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The use of HLA-A*0201 transgenic mice to assess in vivo immunogenicity induced by DNA or peptide immunization has proven to be an excellent model system to identify and study HLA-A*0201 restricted CTL epitopes (47-50). Their specific CTL response was found to correlate well to that of HLA-A*0201 humans (51). Here we show that both DNA and peptide based immunization results in strong HLA-A*0201 restricted CTL responses to specific peptide epitopes. We define several naturally processed HLA-A*0201 presented CTL epitopes of the immunodominant hsp 65 molecule. Despite the strong homology between mycobacterial and eukaryotic hsp65, none of the identified mycobacterial epitopes was identical to the corresponding mouse or human hsp 65 sequences. One of these epitopes, the dominant high affinity Mhsp65(9₃₆₉) epitope is remarkably identical to that of other organisms including a large number of human pathogens such as *M. leprae*, *M. paratuberculosis*, *Brucella abortus*, *Leptospira interrogans*, *Legionella pneumophila*, *Coxiella burnetii*, *Staphylococcus aureus*, *Salmonella typhi*, *Yersinia enterocolitica*, *Neisseria meningitidis*, *Neisseria gonorrhoeae*, *Haemophilus influenzae* and *Pseudomonas aeruginosa*. Of note, this epitope is also strongly homologous to the human sequence, yet it does not induce a cross reactive T cell response. In addition, while the Mhsp65(9₃₆₉) epitope is well generated by human or mouse proteasomes

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the homologous human sequence is most likely not so. This epitope therefore allows further studies of the role of HLA class I restricted CTL mediated immune responses in the protection from a variety of major infectious diseases, and should be considered for use in inducing
5 protective immunity against a broad variety of different pathogens.

BRIEF SUMMARY OF THE INVENTION

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The present invention relates to the use of small epitopic sequences, and sequences having homology thereto, to induce formation of cytotoxic T lymphocyte (CTL) responses to said epitopes as a means of treating and/or preventing infections by microbial agents, especially
15 bacteria, most especially mycobacteria.

It is an object of the present invention to provide epitopic sequences capable of generating specific CTL responses in an animal, such as a human, especially one infected with a microbial agent, or at risk
20 of such infection.

It is also an object of the present invention to provide immunogens, and immunogenic composition, including immunogens that comprise, or consist of, polypeptide structure that themselves contain sequences
25 homologous to the epitopic sequences disclosed according to the invention and wherein such immunogens are useful in eliciting specific CTL responses *in vivo* or *ex vivo*. Such methods may or may not include the use of an antigen presenting cell (APC).

30 It is another object of the present invention to provide vaccines for use in preventing microbial infections using vaccines comprising the

immunogens disclosed according to the present invention, as well as vaccines comprising DNA encoding polypeptides disclosed according to the invention. Such DNA vaccines include plasmid-based vaccines as well as DNA-vaccines wherein the DNA is contained within a virus, such as
5 Adeno-Associated Virus (AAV).

It is a further object of the present invention to provide methods for screening and/or diagnosing microbial-mediated diseases using the immunogens disclosed herein.

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BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Six HLA-A*0201 restricted Mhsp65 derived peptides are CTL epitopes. Mice were immunized with pDNA expressing the Mhsp65. Splenocytes were re-stimulated once in vitro with the relevant peptide as indicated next to x-axis and tested after 5-7 days against the indicated target in a conventional ^{51}Cr release cytotoxicity assay. Similar data were obtained from at least two independent experiments.

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Figure 2. The Mhsp65(9₃₆₉) specific CTL are of high affinity. Peptides specific CTL lines were prepared as described in Fig. 1 and tested against the target cell line T2 which lack the mouse CD8 binding site in a conventional ^{51}Cr release cytotoxicity assay. Similar data were obtained
25 from at least two independent experiments.

Figure 3. Peptide KLAGGVAVL (PGhsp65(9₃₆₉)) can induce specific CTL from the splenocytes of Mhsp65 pDNA immunized mice. A CTL line was prepared by stimulating splenocytes with peptide for five days. Lysis
30 of the JK-A2/Kb cells pulsed with PGhsp65(9₃₆₉) or as control unpulsed or

HIV peptide pulsed cells was measured with a conventional ^{51}Cr release cytotoxicity assay.

Figure 4. a) Peptide immunization with the Mhsp65(9₃₆₉) induce
5 specific CTL that do not cross react with the corresponding sequence
from the human or the mouse hsp65. Mice were primed and boosted after
two weeks with Mhsp65(9₃₆₉) peptide emulsified in IFA. Ten days after
the boosting splenocytes were re-stimulated once in vitro and tested
10 against the indicated target in a conventional ^{51}Cr release cytotoxicity
assay. Similar data were obtained from three independent experiments. b)
Peptide immunization with the Mhsp65(9₃₆₉) induce a specific proliferative
response as compared to immunization with the influenza MP peptide.
Splenocytes were prepared as described in figure 1 and pulsed with
peptides, recombinant Mhsp65 or PPD. Five days later, the cells were
15 pulsed with 1 μCi of ^3H -thymidine, further incubated for 18 hours and
then harvested. The amount of incorporated tritium labeled thymidine is
expressed as count per minute. The SDs were $\leq 10\%$.

Figure 5. Mhsp65(9₃₆₉) specific clones exert specific cytotoxicity
20 and IFN- γ production. Clones were prepared by limiting dilution. a)
Cytotoxicity was measured against Jk-A2/Kb unpulsed or pulsed with
Mhsp65(9₃₆₉) using a conventional ^{51}Cr release assay. IFN- γ production
was measured by ELIspot assay using syngeneic splenocytes as
stimulator and 500 cells from Clone D1 in the presence of the denoted
25 peptide. The number of IFN- γ producing cells is expressed as the number
of spot forming cells (SFC). Peptides were added at different
concentrations as indicated (b). The IFN- γ production is HLA Class I
restricted. The assay was performed as in b in the presence of 1 $\mu\text{g}/\text{ml}$
from the indicated peptide in the absence (open bar) or presence (hatched
30 bar) of the HLA Class I specific antibody W6/32 (c).

Figure 6. Proteasome-mediated digestion of 26-mer substrate peptides containing the Mhsp65(9₃₆₉) epitope (A and C) and the human/murine homologue Huhsp65(9₃₉₆) (B and D). Only the epitope containing fragments with the correct C-terminal residues are depicted.

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The 26-mer Mhsp65(9₃₆₉) peptide:

EKLQERLAK₃₆₉LAGGVAVI₃₇₇KAGAATEVE (SEQ ID NO: 84)

The 26-mer Huhsp65(9₃₉₆) peptide:

10 KEKLNERLAK₃₉₆LSDGVAVL₄₀₄KVGGTSDV (SEQ ID NO: 85)

Both human 20S proteasomes derived from Hela cells (A and B) and mouse 20S proteasomes derived from RMA cells (C and D) were used.

15 Figure 7. a) The detection of Mhsp65(9₃₆₉) specific CTL activity from a human T-cell line generated with peptide pulsed DC as stimulator cells. After several autologous restimulations, the T cells were used as effectors in a ⁵¹Cr release assay, with JY EBV-BLCL as the target cell line. b) Human CD8⁺ CTL are specific to peptides Mhsp65(9₃₆₉) and
20 PGhsp65(9₃₆₉) as measured by there ability to respond with TNF- α release. TNF- α was measured using the TNF- α sensitive cell line WEHI. c, d) Human CD8⁺ CTL are specifically induced to produce IFN- γ (c) and TNF- α (d) by infected but not by non infected monocytes. IFN- γ production was measured using ELISA or WEHI sensitivity assay.

25

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates generally to immunogens and
30 immunogenic compositions, and methods of use therefor, for the

prevention, treatment, and diagnosis of microbial infections, especially bacteria and parasites. The invention discloses peptides, polypeptides, and polynucleotides that can be used to stimulate a CTL response against such microbes.

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The present invention relates to isolated peptide epitopes, especially epitopic peptides, wherein the epitopic portion of the peptide is at least about 8 amino acids in length, preferably about 9 amino acids in length (i.e., nonapeptides), and no longer than about 11 amino acids in length, and having an amino acid sequence at least about 55%, preferably at least about 75% identical to an amino acid sequence selected from the group consisting of the amino acid sequences of SEQ ID NOS: 2, 3, 4, 7, 17 and 31, preferably SEQ ID NO: 4. The epitopic sequences of isolated peptides of the present invention also can differ from the sequences of SEQ ID NO: 2, 3, 4, 7, 17 and 31 by no more than about 2 amino acid residues (see Table 4), with such differences preferably being a conservative amino acid residue. Said isolated peptides are commonly immunogens, or at least can have immunogenic activity, possibly requiring a larger carrier molecule to facilitate such activity, or said peptides may have immunogenic activity when part of a larger structure, such as a polypeptide, other than the mycobacterial heat shock protein 65 (Mhsp65) itself or the human heat shock protein 65. Such peptides may also have immunogenic activity when part of a composition containing one or more of said epitopic peptides, which may be present in any combination and with each such peptide being present in one or more copies.

Thus, the epitopic, or immunogenic, peptides (SEQ ID NOS: 2, 3, 4, 7, 17 and 31) of the present invention may be natural peptides, or they may be produced by synthetic or recombinant methodologies that are well known and clear to the skilled artisan (Grant, G. A., Synthetic Peptides: A

User's Guide, 1992, W. H. Freeman and Company, New York; Coligan, J. E. et al, Current Protocols in Protein Science, 1999, John Wiley & Sons, Inc., New York). Besides peptides comprising SEQ ID NOs: 2, 3, 4, 7, 17 and 31, the immunogens of the present invention may also comprise one or more other immunogenic peptides that are known to be associated with the infectious microbes disclosed herein, especially those demonstrated to cross-react with the epitopes of the invention (see Table 3) and which may stimulate a CTL response whereby the immunogenic peptides associate with HLA IA. Said immunogenic peptides could be in the form of a pool of peptides, or a composition comprising one or more of the immunogenic peptides, each present in almost any ratio of concentration relative to the others, including pools or compositions containing copies of one of the immunogenic peptides, or possibly 2, or 3, or more, each present in a concentration either related or unrelated to that of each, or some, or all of the other peptides. Such compositions can, therefore, be homogeneous or heterogeneous with respect to the individual immunogenic peptide components present therein, having only one or more than one of such peptides. For example, an isolated peptide of the present invention can have the sequence of SEQ ID NO: 2, 3, 4, 7, 17 and 31, especially 4.

The immunogenic peptides of the present invention may likewise be represented within an oligopeptide or polypeptide that comprises a peptide epitope portion of at least one of SEQ ID NO: 2, 3, 4, 7, 17 and 31. The oligopeptides and polypeptides may be derived by fractionation of the naturally occurring Mhsp65 protein by methods such as protease treatment, or they may be produced by recombinant or synthetic methodologies that are well known and clear to the skilled artisan (Ausubel, F. M. et al, Current Protocols in Molecular Biology, 1999, John Wiley & Sons, Inc., New York; Coligan, J. E. et al, Current Protocols in Protein Science, 1999, John Wiley & Sons, Inc., New York; Molecular

Cloning: A Laboratory Manual, 1989, Cold Spring Harbor Laboratory Press, Cold Spring Harbor). The polypeptide may comprise : a large portion of the Mhsp65-derived protein; fragments of the Mhsp65-derived protein that comprise at least one of SEQ ID NOs: 2, 3, 4, 7, 17 and 31; a
5 recombinant or synthetic polypeptide that comprises at least one of SEQ ID NOs: 2, 3, 4, 7, 17 and 31; any other sequences or combination of sequences derived from the Mhsp65 protein such as a sequence comprising SEQ ID NO: 4. Thus, oligopeptides and polypeptides of the present invention may have one, two, three, or more such immunogenic
10 peptides within the amino acid sequence of said oligopeptides and polypeptides, and said immunogenic peptides, or epitopes, may be the same or may be different, or may have any number of such epitopes wherein some of them are identical to each other (in amino acid sequence) while others within the same polypeptide sequence are
15 different from each other and said epitopes may occur in any order within said immunogenic polypeptide sequence. The location, arrangement, and ordering of the immunogenic peptides within the sequence of an immunogenic oligopeptide or polypeptide of the present invention will probably, although not necessarily, affect the ability of the various
20 epitopes to be processed from the oligopeptide or polypeptide and to consequently be available to bind to the HLA IA molecules. Thus, the location, arrangement, and ordering of these immunogenic peptides affords the user, either researcher or clinician, with the opportunity to regulate such interaction, or interactions, on the basis of the sequence of
25 the immunogenic oligopeptide or polypeptide generated according to the methods disclosed herein.

Thus, in one embodiment, the present invention relates to an isolated peptide of at least 8 amino acid residues in length and having an amino acid sequence at least 85% identical to the sequence selected from
30 the group consisting of SEQ ID NOS: 2, 3, 4, 7, 17 and 31. In another

mbodiment, the present invention is directed to an isolated peptide epitope of no more than about 11 amino acids in length comprising a sequence selected from the group consisting of SEQ ID NOS: 2, 3, 4, 7, 17 and 31. Specific embodiments of the invention disclosed herein relate to an isolated nonapeptide having an amino acid sequence selected from the group consisting of SEQ ID NOS: 2, 3, 4, 7, 17 and 31 and an isolated nonapeptide having a sequence differing by no more than 2 amino acids from a sequence selected from the group consisting of SEQ ID NOS: 2, 3, 4, 7, 17 and 31 and especially wherein in the latter embodiment, the amino acid difference is a substitution of one conservative amino acid for another.

Conservative substitutions are herein defined as exchanges within one of the following five groups: Group 1—small aliphatic, nonpolar or slightly polar residues (Ala, Ser, Thr, Pro, Gly); Group 2—polar, negatively charged residues and their amides (Asp, Asn, Glu, Gln); Group 3—polar, positively charged residues (His, Arg, Lys); Group 4—large, aliphatic, nonpolar residues (Met, Leu, Ile, Val, Cys); and Group 4—large, aromatic residues (Phe, Tyr, Trp).

Where the immunogen comprises two or more immunogenic epitopes, or epitopic peptides, they may be linked directly together, or through a spacer or linker, to form a larger structure, such as an oligopeptide, or polypeptide, or some other polymeric structure. The epitopic peptides may therefore be linked by any and all means that can be devised by the chemist so long as the immunogenic activity of the overall structure or complex is maintained or, at least, not reduced below a level useful for the methods of the invention (i.e., especially where said immunogenic activity comprises being capable of eliciting a CTL response). Likewise, the immunogenic peptide may be linked directly to, or through a spacer or linker to: an immunogenic carrier such as serum

albumin, tetanus toxoid, keyhole limpet hemocyanin, dextran, or a recombinant virus particle; an immunogenic peptide known to stimulate a T helper cell type immune response; a cytokine such as interferon gamma or GMCSF; a targeting agent such as an antibody or receptor ligand; a stabilizing agent such as a lipid; or a conjugate of a plurality of epitopes to a branched lysine core structure, such as the so-called "multiple antigenic peptide" described in (Posnett, D. N. et al., J.Biol.Chem., 263:1719-1725, (1988)); a compound such as polyethylene glycol to increase the half life of the peptide; or additional amino acids such as a leader or secretory sequence, or a sequence employed for the purification of the mature sequence. Spacers and linkers are typically comprised of relatively small, neutral molecules, such as amino acids and which are substantially uncharged under physiological conditions. Such spacers are typically selected from the group of nonpolar or neutral polar amino acids, such as glycine, alanine, serine and other similar amino acids. Such optional spacers or linkers need not be comprised of the same residues and thus may be either homo- or hetero-oligomers. When present, such linkers will commonly be of length at least one or two, commonly 3, 4, 5, 6, and possibly as much as 10 or even up to 20 residues (in the case of amino acids). In addition, such linkers need not be composed of amino acids but any oligomeric structures will do as well so long as they provide the correct spacing so as to optimize the desired level of immunogenic activity of the immunogens of the present invention. The immunogen may therefore take any form that is capable of eliciting a CTL response, especially a CD8⁺ CTL response.

In addition, the immunogenic peptides of the present invention may be part of an immunogenic structure via attachments other than conventional peptide bonds. Thus, any manner of attaching the peptides of the invention to an immunogen of the invention, such as an immunogenic polypeptide as disclosed herein, could provide an immunogenic structure as

claimed herein. Thus, immunogens, such as peptides of the invention, are structures that contain the peptides disclosed according to the present invention but such immunogenic peptides may not necessarily be attached thereto by the conventional means of using ordinary peptide bounds. The
5 immunogens of the present invention simply contain such peptides as part of their makeup, but how such peptides are to be combined to form the final immunogen is left to the talent and imagination of the user and is in no way restricted or limited by the disclosure contained herein.

The peptides useful in practicing the methods of the present
10 invention need not be the optimal peptides for stimulating a CTL response. See, for example, (Parkhurst, M. R. et al., J.Immunol., 157:2539-2548, (1996); Rosenberg, S. A. et al., Nat.Med., 4:321-327, (1998)). Thus, there can be utility in modifying a peptide, such that it more readily induces a CTL response.

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Thus, the present invention is directed to the use of specific peptide epitopes to induce a cytotoxic T lymphocyte (CTL) response in an animal. Mycobacteria are intracellular pathogens which reside in macrophages (62). Vaccines targeting mycobacterial pathogens are
20 therefore crucially dependent on the triggering of a T cell mediated immune response. Accordingly, the definition of MHC class I restricted CD8⁺ CTL that are able to recognize mycobacteria derived epitopes is of central importance for development of vaccines and diagnostic reagents to be used e.g. for DTH testing. In addition, elucidation of such epitopes
25 provide a better understanding of the role of MHC class-I restricted CTL mediated immune responses in the protection from and susceptibility to infectious diseases. Here, we have used pDNA vaccination of HLA-A*0201/Kb transgenic mice with a full length mycobacterial gene to define the first human CTL epitopes from an immunodominant
30 mycobacterial antigen, the hsp65 (63), and demonstrate that T cells

specific for at least one of these high affinity MHC binding pitopes (Mhsp65(9₃₆₉)) are part of the T cell repertoire in HLA-A*0201 positive human beings. The same epitope was also shown to be expressed in monocytes/macrophages infected with live BCG, a finding of crucial importance in relation to the potential use of this epitope in protective vaccines. We also demonstrate the efficiency of the pDNA and peptide vaccination methods in activating cytotoxic, proliferative and cytokine producing T cell responses against a mycobacterial antigen, a finding with considerable clinical implications.

10

The immunogenic peptides and polypeptides of the invention can be prepared synthetically, by recombinant DNA technology, or they can be isolated from natural sources such as microbial cells expressing the heat shock protein 65, where said protein is homologous, especially highly homologous, to Mhsp65.

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The peptides can be synthesized in solution or on a solid support in accordance with conventional techniques. Various automated peptide synthesizers are commercially available and can be used in accordance with known protocols. See, for example, (Grant, G. A., Synthetic Peptides: A User's Guide, 1992, W. H. Freeman and Company, New York; Coligan, J. E. et al, Current Protocols in Protein Science, 1999, John Wiley & Sons, Inc., New York). Fragments of the polypeptide of the invention can also be synthesized as intermediates in the synthesis of a larger polypeptide.

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Recombinant DNA technology may be employed wherein a nucleotide sequence which encodes an immunogenic peptide or polypeptide of interest is inserted into an expression vector, transformed or transfected into an appropriate host cell, and cultivated under conditions suitable for expression. These procedures are well known in the art to the skilled artisan, as described in (Coligan, J. E. et al, Current Protocols in

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Immunology, 1999, John Wiley & Sons, Inc., New York; Ausubel, F. M. et al, Current Protocols in Molecular Biology, 1999, John Wiley & Sons, Inc., New York; Molecular Cloning: A Laboratory Manual, 1989, Cold Spring Harbor Laboratory Press, Cold Spring Harbor). Thus, recombinantly
5 produced peptides or polypeptides can be used as the immunogens of the invention.

The coding sequences for peptides of the length contemplated herein can be synthesized on commercially available automated DNA synthesizers using protocols that are well known in the art. See for example, (Grant, G.
10 A., Synthetic Peptides: A User's Guide, 1992, W. H. Freeman and Company, New York; Coligan, J. E. et al, Current Protocols in Protein Science, 1999, John Wiley & Sons, Inc., New York). The coding sequences can also be modified such that a peptide or polypeptide will be produced that incorporates a desired amino acid substitution. The coding
15 sequence can be provided with appropriate linkers, be ligated into suitable expression vectors that are commonly available in the art, and the resulting DNA or RNA molecule can be transformed or transfected into suitable hosts to produce the desired fusion protein. A number of such vectors and suitable host systems are available, and their selection is left to the skilled
20 artisan. For expression of the fusion proteins, the coding sequence will be provided with operably linked start and stop codons, promoter and terminator regions, and a replication system to provide an expression vector for expression in the desired host cell. For example, promoter sequences compatible with bacterial hosts are provided in plasmids containing
25 convenient restriction sites for insertion of the desired coding sequence. The resulting expression vectors are transformed into suitable bacterial hosts. Of course, yeast, insect, and mammalian host cells may also be used, employing suitable vectors and control sequences.

In accordance with the present invention, six HLA-A*0201 restricted CTL epitopes were identified, and at least one of these, the Mhsp65(9₃₆₉) (SEQ ID NO: 4), is recognized with high affinity based on its independence of the CD8-MHC class I interaction to induce efficient specific cytotoxicity. Because all 6 epitopes were generated by priming mice with i.m. immunization with the full length Mhsp65 gene, the injected plasmid must have either directly transfected bone marrow derived dendritic cells or, alternatively, the Mhsp65 could have been expressed in myocytes followed by indirect processing by professional antigen presenting cells (APC). In either case, this has resulted in the generation of a number of naturally processed HLA-A*0201 associated peptide epitopes that were able to activate specific CTL including the six epitopes disclosed herein.

DNA immunization is a novel approach to induce a specific immune responses to microorganisms and tumors (65), and has recently been reported to generate MTB specific CTL responses in mice (45, 66). This response was also shown to mediate protection when used in adoptive CTL transfer studies (30). DNA immunization with the full length Mhsp65 not only elicits specific T-cell responses but also specific antibody responses (J. Charo et al, manuscript in preparation). In view of this efficient activation of both the cellular and the humoral immune system after pDNA hsp65 immunization it is likely that this is an efficient way of inducing protective as well as therapeutic immunity (67).

Of major importance is the observation that the Mhsp65(9₃₆₉) specific human CTL can respond specifically to BCG infected monocytes. This represents the first evidence of a mycobacterial antigen that presumably accesses the MHC-I processing and presentation pathway in human macrophages, a finding that is supported also by the proteasome digestion data. Macrophage activation by T helper 1 (Th1) cytokines is

essential for resistance to tuberculosis (68-71). Studies on IFN- γ and IFN- γ receptor knockout mice (72) and on IFN- γ R or IL-12R β 1 deficient patients (73) demonstrated the importance of this cytokine in the protective immunity against mycobacterial infection. Similar studies using TNF- α receptor knockout mice have also established the importance of TNF- α in the protection against mycobacteria (74). Our data on the induction of these two cytokines upon stimulation with Mhsp65(9₃₆₉) suggests that this immune response could lead to both macrophage activation and granuloma formation.

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As the high-affinity Mhsp65(9₃₆₉) epitope is also recognized by human CTL, it appears as a particularly attractive candidate to be included in a peptide or pDNA epitope expressing mini-gene based vaccine. Furthermore, this peptide has a remarkable identity with the corresponding region of hsp60 from several pathogens including *M. leprae*, *M. paratuberculosis*, *Coxiella burnetii*, *Brucella abortus*, *Leptospira interrogans*, *Legionella pneumophila*, *Staphylococcus aureus*, *Salmonella typhi*, *Yersinia enterocolitica*, *Neisseria meningitidis*, *Neisseria gonorrhoeae*, *Haemophilus influenzae* and *Pseudomonas aeruginosa*. Also the rest of the immunogenic peptides described here have identical sequences in hsp65 from a number of pathogens. This might explain in part the cross-protection described earlier between different bacteria (75) and more importantly suggests a simple approach for vaccination against these diseases based on the use of a single epitope. The use of this epitope or T cell clones that specifically recognize it should help to extend our knowledge about the role of MHC class I restricted CTL mediated immune responses in the pathogenesis caused by or associated with these bacteria. This would be particularly beneficial in the cases of mycobacteria and yersinia where a role for CD8⁺ T cell mediated protection has already been described (13).

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Mhsp65 shares a strong homology with human and mouse hsp65, and immunity to hsp65 has been implicated in several autoimmune conditions, including rheumatoid arthritis, diabetes and atherosclerosis (75-78). This has dampened the enthusiasm for using mycobacterial vaccines based on the full-length hsp65 molecule. Yet, when we here immunize with the full length Mhsp65 gene, the resulting immune response was solely directed against CTL epitopes with sequences that are not identical to human and mouse hsp65. Among these six epitopes defined here, the Mhsp65(9₃₆₂) is the one that has the most homology to the corresponding sequence from the mouse and the human hsp65 (Huhsp65(9₃₉₆)). However, a CTL line that specifically killed target cells pulsed with Mhsp65(9₃₆₂) did not cross react with the same target cells when pulsed with the Huhsp65(9₃₉₆) epitope.

To further demonstrate the processing of mycobacterial and human hsp 65 sequences, purified proteasomes of human and mouse origin were used to show that these proteasomes efficiently generated the dominant Mycobacterial hsp65(9₃₆₉). In contrast, another high affinity HLA-A*0201 binding peptide, not recognized by CTL, or the human/mouse hsp 65, were not processed by these proteasomes. A well-defined cleavage site behind the C-terminus of the (9₃₆₉) epitope was used, whereas the N-terminus was not as clearly defined. It has been reported for several CTL epitopes (79-81) that proteasomes properly define the C-terminus of MHC class I restricted epitopes, whereas the N-terminus of epitope-precursor peptides is frequently elongated by one or more amino acids. This requires N-terminal trimming events (81-83), but can allow more efficient TAP-translocation of epitopes that are not as efficiently translocated as the MHC class I presented minimal peptide sequence (84, 85).

The relevant precursor fragments of the human/murine homologue sequence of the Mhsp65(9₃₉₆) epitope could not be detected in the

digests of the human homologue peptide. These experiments suggest that this peptide is not generated in vivo. Sequence alterations of only one amino acid within or outside a CTL epitope can already disrupt CTL epitope processing (85, 86). The observed lack of processing by itself, rather than the absence of autoreactive T cells by tolerization events, is sufficient to explain the lack of responsiveness to a potential self-epitope. Comparison of human and mouse proteasome digestion products (Fig 6) showed that proteasome cleavage is strikingly similar. Apparently proteasome function in mammals is highly conserved. This aspect makes HLA-transgenic mice an even more powerful tool to identify human CTL epitopes and to study relevant immune responses in animal models.

Nevertheless, to avoid the possibility of evoking an autoimmune reaction, strategies to vaccinate against MTB might include immunization based on peptides or pDNA mini-genes expressing these epitopes which are not shared between human and bacterial hsp65. In particular, those epitopes that have earlier been reported to induce cross reactivity such as the HLA-DR or DQ presented ones that map to the 241-255 and 241-265 stretches respectively should be avoided (87). As we have here shown that immunization with peptide Mhsp65(9₃₆₉) has resulted in a specific immune responses, it would be reasonable to suggest the use of this peptide as a preventive vaccine against the above mentioned pathogens, where a protective immune response could be CTL dependent. Alternatively it is possible to use this epitope in a therapeutic or a diagnostic setting, such as the basis for a vaccine.

The present invention further relates to a polypeptide comprising an epitopic sequence selected from sequences homologous (differing by no more than 2 amino acid residues) from the sequences of SEQ ID NO: 2, 3, 4, 7, 17 and 31, as well as fragments, analogs and derivatives of such

polypeptide, but not including the full length Mhsp65 or human heat shock protein 65 (Huhsp65).

5 The terms "fragment," "derivative" and "analog" when referring to the polypeptide, means a polypeptide which retains essentially the same biological function or activity as such polypeptide. Thus, an analog includes a proprotein which can be activated by cleavage of the proprotein portion to produce an active mature polypeptide. Such fragments, derivatives and analogs must have sufficient similarity to the polypeptides comprising
10 epitopes homologous to SEQ ID NO: 2, 3, 4, 7, 17 and 31 so that activity of the native polypeptide is retained.

The polypeptide of the present invention may be a recombinant polypeptide, a natural polypeptide or a synthetic polypeptide, preferably a
15 recombinant polypeptide.

The fragment, derivative or analog of such a polypeptide may be (i) one in which one or more of the amino acid residues are substituted with a conserved or non-conserved amino acid residue (preferably a conserved
20 amino acid residue) and such substituted amino acid residue may or may not be one encoded by the genetic code, or (ii) one in which one or more of the amino acid residues includes a substituent group, or (iii) one in which the mature polypeptide is fused with another compound, such as a compound to increase the half-life of the polypeptide (for example,
25 polyethylene glycol), or (iv) one in which the additional amino acids are fused to the mature polypeptide, such as a leader or secretory sequence or a sequence which is employed for purification of the mature polypeptide or a proprotein sequence. Such fragments, derivatives and analogs are deemed to be within the scope of those skilled in the art from the teachings
30 herein.

The polypeptides and polynucleotides of the present invention are preferably provided in an isolated form, and preferably are purified to homogeneity.

5 The term "isolated" means that the material is removed from its original environment (e.g., the natural environment if it is naturally occurring). For example, a naturally-occurring polynucleotide or polypeptide present in a living animal is not isolated, but the same polynucleotide or polypeptide, separated from some or all of the coexisting materials in the
10 natural system, is isolated. Such polynucleotides could be part of a vector and/or such polynucleotides or polypeptides could be part of a composition, and still be isolated in that such vector or composition is not part of its natural environment.

15 As known in the art "similarity" between two polypeptides is determined by comparing the amino acid sequence and its conserved amino acid substitutes of one polypeptide to the sequence of a second polypeptide.

20 In accordance with the present invention, the term "percent identity" or "percent identical," when referring to a sequence, means that a sequence is compared to a claimed or described sequence after alignment of the sequence to be compared (the "Compared Sequence") with the described or claimed sequence (the "Reference Sequence"). The Percent Identity is then
25 determined according to the following formula:

$$\text{Percent Identity} = 100 [1 - (C/R)]$$

wherein C is the number of differences between the Reference Sequence
30 and the Compared Sequence over the length of alignment between the Reference Sequence and the Compared Sequence wherein (i) each base or

amino acid in the Reference Sequence that does not have a corresponding aligned base or amino acid in the Compared Sequence and (ii) each gap in the Reference Sequence and (iii) each aligned base or amino acid in the Reference Sequence that is different from an aligned base or amino acid in the Compared Sequence, constitutes a difference; and R is the number of bases or amino acids in the Reference Sequence over the length of the alignment with the Compared Sequence with any gap created in the Reference Sequence also being counted as a base or amino acid.

10 If an alignment exists between the Compared Sequence and the Reference Sequence for which the percent identity as calculated above is about equal to or greater than a specified minimum Percent Identity then the Compared Sequence has the specified minimum percent identity to the Reference Sequence even though alignments may exist in which the
15 hereinabove calculated Percent Identity is less than the specified Percent Identity.

Fragments or portions of the polypeptides of the present invention may be employed for producing the corresponding full-length polypeptide by peptide synthesis; therefore, the fragments may be employed as
20 intermediates for producing the full-length polypeptides. Fragments or portions of the polynucleotides of the present invention may be used to synthesize full-length polynucleotides of the present invention.

As used herein, the terms "portion," "segment," and "fragment,"
25 when used in relation to polypeptides, refer to a continuous sequence of residues, such as amino acid residues, which sequence forms a subset of a larger sequence. For example, if a polypeptide were subjected to treatment with any of the common endopeptidases, such as trypsin or chymotrypsin, the oligopeptides resulting from such treatment would
30 represent portions, segments or fragments of the starting polypeptide. This means that any such fragment will necessarily contain as part of its

amino acid sequence a segment, fragment or portion, that is substantially identical, if not exactly identical, to a sequence of SEQ ID NOS: 2, 3, 4, 7, 17 and 31. When used in relation to polynucleotides, such terms refer to the products produced by treatment of said polynucleotides with any of
5 the common endonucleases.

In accordance with the foregoing, the present invention relates to an isolated peptide of at least 8 amino acid residues in length (see Table 1) and having an amino acid sequence at least 75% identical to the sequence selected from the group consisting of SEQ ID NOS: 2, 3, 4, 7,
10 17 and 31, preferably 4.

In another aspect, the present invention relates to an isolated peptide of no more than about 11 amino acids in length comprising a sequence selected from the group consisting of SEQ ID NOS: 2, 3, 4, 7, 17 and 31.

15 In one aspect, the present invention relates to an isolated nonapeptide having an amino acid sequence selected from the group consisting of SEQ ID NOS: 2, 3, 4 and 31.

In a separate embodiment, the present invention relates to an isolated nonapeptide having a sequence differing by no more than 1
20 amino acid from a sequence selected from the group consisting of SEQ ID NOS: 2, 3, 4 and 31, preferably wherein the amino acid difference is a substitution of one conservative amino acid for another.

In another aspect the present invention relates to an immunogen comprising one or more isolated peptides as disclosed herein, especially
25 peptides with at least 85% sequence homology with the peptides of selected from the group consisting of the peptides of SEQ ID NOS: 2, 3, 4, 7, 17 and 31, especially an immunogen comprising a polypeptide

containing the amino acid sequence of SEQ ID NO: 4, wherein said polypeptide is not the mycobacterial heat shock protein 65 or the human protein.

5 In one embodiment, the present invention relates to an immunogen comprising a polypeptide, other than mycobacterial heat shock protein 65 (Mhsp65) or Huhsp65 (or polypeptides highly homologous thereto), wherein said polypeptide comprises one or more epitopes selected from the peptides of SEQ ID NOS: 2, 3, 4, 7, 17 and 31 and wherein each said epitopic peptide may be present in one or more copies.

10 In another aspect the present invention relates to an immunogen comprising a polynucleotide sequence coding for at least one cytotoxic T lymphocyte (CTL) epitope, wherein said epitope is selected from the peptides of SEQ ID NOS: 2, 3, 4, 7, 17 and 31 and wherein each said epitopic peptide may be present in one or more copies.

15 The present invention also relates to a method for inducing a CTL response *in vitro* that is specific for an infectious microbe expressing at least one of the epitopes of SEQ ID NO: 2, 3, 4, 7, 17 and 31 wherein the method comprises contacting a precursor CTL with an immunogenic peptide selected from a group comprising the peptide immunogens
20 disclosed herein, especially those described above, under conditions that generate a CTL response to the microbe.

The present invention also relates to a method for inducing a CTL response *in vitro* that is specific for an infectious microbe expressing at least one of the epitopes of SEQ ID NO: 2, 3, 4, 7, 17 and 31 wherein
25 the method comprises contacting a precursor CTL with an antigen presenting cell that has exogenously acquired one or more of the immunogens disclosed herein.

The present invention also relates to a method of inducing a CTL response *in vitro* that is specific for an infectious microbe expressing at least one of the epitopes of SEQ ID NO: 2, 3, 4, 7, 17 and 31 wherein the method comprises contacting a precursor CTL with an antigen
5 presenting cell that is expressing a polynucleotide encoding an immunogen, or epitope, according to the invention but not including the polypeptide expressing the entire Mshp65 gene or Huhsp65.

In another aspect, the present invention is directed to a method of treating a subject infected with an infectious microbe expressing at least
10 one of the epitopes of SEQ ID NO: 2, 3, 4, 7, 17 and 31 wherein the CTLs induced by the methods disclosed herein are administered in an amount sufficient to destroy the infectious microbes through direct lysis or to effect the destruction of the infectious microbes indirectly through the elaboration of cytokines.

15 The method of the present invention is well suited to situations wherein the infectious microbe is selected from the group consisting of Mycobacteria, Chlamydia, Trypanosoma, Helicobacter, Leishmania, Trichomonas, Synechococcus, and Cowdria (as evidenced by the cross-reactivity disclosed in Table 3), as well as *M. leprae*, *M. paratuberculosis*,
20 *Brucella abortus*, *Leptospira interrogans*, *Legionella pneumophila*, *Coxiella burnetii*, *Staphylococcus aureus*, *Salmonella typhi*, *Yersinia enterocolitica*, *Neisseria meningitidis*, *Neisseria gonorrhoeae*, *Haemophilus influenzae* and *Pseudomonas aeruginosa*, and including such organisms as *Mycobacterium tuberculosis*, *Trichomonas vaginalis*, *Synechococcus vulcanis*, and *Cowdria ruminatum*. Such organisms also include any of
25 those mentioned in Table 4.

In a preferred embodiment, the infectious microbe is a bacterium, especially a bacterium is of the genus *Mycobacterium*, most especially *Mycobacterium tuberculosis*.

In one aspect, the present invention relates to a method for inducing a CTL response in a subject comprising administering an immunogen comprising a peptide having an amino acid sequence selected from the group consisting of (a) SEQ ID NO: 2, 3, 7, 17 and 31; (b) a sequence differing from (a) by at least one but no more than 2 amino acids; (c) SEQ ID NO: 4; (d) a sequence differing from (c) by at least one but no more than 2 amino acids at least one CTL epitope, and wherein said immunogen is other than a bacterial heat shock protein 65 and wherein said peptide is other than a peptide contained in human heat shock protein 65.

In another aspect the present invention relates to a method for inducing a CTL response in a subject, whereby the method comprises administering at least one CTL epitope, wherein said epitope is selected from a group consisting of the immunogens disclosed herein to an HLA IA positive subject, in an amount sufficient to induce a CTL response to infectious microbes expressing at least one of the epitopes of SEQ ID NO: 2, 3, 4, 7, 17 and 31, or epitopic sequences homologous thereto, such as those differing therefrom by no more than 2 amino acid residues but wherein said immunogen is other than a bacterial heat shock protein 65 and wherein said peptide is other than a peptide contained in human heat shock protein 65.

The present invention also relates to a method for inducing a CTL response in a subject, whereby the method comprises administering an immunogen as disclosed herein to a subject, in an amount sufficient to induce a CTL response to infectious microbes expressing at least one of the epitopes of SEQ ID NO: 2, 3, 4, 7, 17 and 31 but wherein said immunogen is other than a bacterial heat shock protein 65 and wherein said peptide is other than a peptide contained in human heat shock protein 65.

In accordance with the present invention, a preferred embodiment for inducing the CTL responses disclosed herein involves situations wherein said induced CTL response is a CD8⁺ CTL response.

5 The present invention further relates to use of polypeptides (other than Mhsp65 itself, or a protein highly homologous to Huhsp65) comprising the peptide epitopes disclosed herein as the basis for a vaccine or for the generation of protective antibodies and CTLs. One means for accomplishing this is by introducing a cell that expresses a
10 protein or polypeptide that incorporates an epitopic sequence homologous to the epitopic sequences disclosed herein, especially SEQ ID NO: 2, 3, 4, 7, 17 and 31, most especially 4, and wherein such homologous sequences will differ therefrom by no more than about 2 amino acid residues. Some such sequences are described in Table 4. The
15 immunogens of the present invention comprise peptide epitopes wherein said immunogen is other than a bacterial heat shock protein 65 and wherein said peptide is other than a peptide contained in human heat shock protein 65.

 The pharmaceutical compositions useful herein also contain a
20 pharmaceutically acceptable carrier, including any suitable diluent or excipient, which includes any pharmaceutical agent that does not itself induce the production of antibodies harmful to the individual receiving the composition, and which may be administered without undue toxicity. Pharmaceutically acceptable carriers include, but are not limited to, liquids
25 such as water, saline, glycerol and ethanol, and the like, including carriers useful in forming sprays for nasal and other respiratory tract delivery or for delivery to the ophthalmic system. A thorough discussion of pharmaceutically acceptable carriers, diluents, and other excipients is presented in REMINGTON'S PHARMACEUTICAL SCIENCES (Mack Pub.
30 Co., N.J. current edition).

Vaccine compositions may further incorporate additional substances to stabilize pH, or to function as adjuvants, wetting agents, or emulsifying agents, which can serve to improve the effectiveness of the vaccine. The
5 vaccine compositions of the present invention may further incorporate cytokines or co-stimulatory molecules as proteins or as plasmids coding for these proteins.

The present invention also relates to use of vaccines comprising
10 plasmids as vaccination agent (plasmid vaccines or pDNA vaccines) and which encode immunogens and polypeptides disclosed according to the invention, including plasmids that comprise DNA encoding active fragments of the immunogens and polypeptides disclosed according to the invention as well as DNA encoding minigenes of the active peptide epitopes. Such
15 plasmid-based vaccines are commonly administered by subcutaneous, intramuscular, intradermal or other means of delivery, either through use of a needle or through ballistic means of administration, including, but not limited to, use of a gene gun.

20 Based on cytotoxicity assays, an epitope is considered substantially identical to the reference peptide if it has at least 10% of the antigenic activity of the reference peptide as defined by the ability of the substituted peptide to reconstitute the epitope recognized by a CTL in comparison to the reference peptide. Thus, when comparing the lytic activity in the linear
25 portion of the effector:target curves with equimolar concentrations of the reference and substituted peptides, the observed percent specific killing of the target cells incubated with the substituted peptide should be equal to that of the reference peptide at an effector:target ratio that is no greater than 10-fold above the reference peptide effector:target ratio at which the
30 comparison is being made.

Preferably, when the CTL specific for a peptide of SEQ ID NO: 2, 3, 4, 7, 17 and 31 are tested against the substituted peptides, the peptide concentration at which the substituted peptides achieve half the maximal increase in lysis relative to background is no more than about 1 mM, preferably no more than about 1 μ M, more preferably no more than about 1 nM, and still more preferably no more than about 100 pM, and most preferably no more than about 10 pM. It is also preferred that the substituted peptide be recognized by CTLs from more than one individual, at least two, and more preferably three individuals.

10

The immunogenic peptides of the present invention may be used to elicit CTLs *ex vivo* from either healthy individuals or from patients exhibiting symptoms of infection with a microbial organism, such as an individual with tuberculosis. Such responses are induced by incubating in tissue culture the individual's CTL precursor lymphocytes together with a source of antigen presenting cells and the appropriate immunogenic peptide. Examples of suitable antigen presenting cells include dendritic cells, macrophages, and activated B cells. Typically, the peptide at concentrations between 10 and 40 μ g/ml, would be pre-incubated with the antigen presenting cells for periods ranging from 1 to 18 hrs. β_2 -microglobulin (4 μ g/ml) can be added during this time period to enhance binding. The antigen presenting cells may also be held at room temperature during the incubation period (Ljunggren, H.-G. et al., *Nature*, 346:476-480, (1990)) or pretreated with acid (Zeh, H. J., III et al., *Hum.Immunol.*, 39:79-86, (1994)) to promote the generation of denatured class I MHC molecules which can then bind the peptide. The precursor CTLs (responders) are then added to the antigen presenting cells to which the immunogenic peptide has bound (stimulators) at responder to stimulator ratios of between 5:1 and 50:1, and most typically between 10:1 and 20:1. The co-cultivation of the cells is carried out at 37°C in RPMI 1640, 10% fetal bovine serum, 2 mM L-glutamine,

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and IL-2 (5-20 Units/ml). Other cytokines, such as IL-1, IL-7, and IL-12 may also be added to the culture. Fresh IL-2-containing media is added to the cultures every 2-4 days, typically by removing one-half the old media and replenishing it with an equal volume of fresh media. After 7-10 days, and every 7-10 days thereafter, the CTL are restimulated with antigen presenting cells to which immunogenic peptide has been bound as described above. Fresh IL-2-containing media is added to the cells throughout their culture as described above. Three to four rounds of stimulation, and sometimes as many five to eight rounds of stimulation, are required to generate a CTL response that can then be measured *in vitro*. The above described protocol is illustrative only and should not be considered limiting. Many *in vitro* CTL stimulation protocols have been described and the choice of which one to use is well within the knowledge of the skilled artisan. The peptide-specific CTL can be further expanded to large numbers by treatment with anti-CD3 antibody. For example, see (Riddell, S. R. and Greenberg, P. D., J.Immunol.Methods, 128:189-201, (1990); Walter, E. A. et al., N.Engl.J.Med., 333:1038-1044, (1995)).

Antigen presenting cells that are to be used to stimulate a CTL response are typically incubated with peptide of an optimal length, most commonly a nonapeptide, that allows for direct binding of the peptide to the class I MHC molecule without additional processing. Larger oligopeptides and polypeptides are generally ineffective in binding to class I MHC molecules as they are not efficiently processed into an appropriately sized peptide in the extracellular milieu. There are a variety of approaches that are known in the art, however, that allow oligopeptides and polypeptides to be exogenously acquired by a cell, which then allows for their subsequent processing and presentation by a class I MHC molecule. Representative, but non-limiting, examples of such approaches include electroporation of the molecules into the cell (Harding,

C. H. III, Eur.J.Immunol., 22:1865-1869, (1992)), encapsulation of the molecules in liposomes which are fused to the cells of interest (Reddy, R. et al., J.Immunol.Methods, 141:157-163, (1991)), or osmotic shock in which the molecules are taken up via pinocytosis (Moore, M. W. et al.,
5 Cell, 54:777-785, (1988)). Thus, oligopeptides and polypeptides that comprise one or more of the peptides of the invention can be provided to antigen presenting cells in such a fashion that they are delivered to the cytoplasm of the cell, and are subsequently processed to allow presentation of the peptides.

10 Antigen presenting cells suitable for stimulating an *in vitro* CTL response that is specific for one or more of the peptides of the invention can also be prepared by introducing polynucleotide vectors encoding the sequences into the cells. These polynucleotides can be designed such that they express only a single peptide of the invention, multiple peptides of
15 the invention, or even a plurality of peptides of the invention. There are a variety of approaches that are known in the art, that allow polynucleotides to be introduced and expressed in a cell, thus providing one or more peptides of the invention to the class I MHC molecule binding pathway. Representative, but non-limiting, examples of such approaches
20 include the introduction of plasmid DNA through particle-mediated gene transfer or electroporation (Tuting, T. et al., J.Immunol., 160:1139-1147, (1998)), or the transduction of cells with an adenovirus expressing the polynucleotide of interest (Perez-Diez, A. et al., Cancer Res., 58:5305-5309, (1998)). Thus, oligonucleotides that code for one or more of the
25 peptides of the invention can be provided to antigen presenting cells in such a fashion that the peptides associate with class I MHC molecules and are presented on the surface of the antigen presenting cell; and consequently are available to stimulate a CTL response.

By preparing the stimulator cells used to generate an *in vitro* CTL response in different fashions, it is possible to control the peptide specificity of CTL response. For example, the CTLs generated with a particular peptide will necessarily be specific for that peptide. Likewise, CTLs that are generated with a polypeptide or polynucleotide expressing or coding for particular peptides will be limited to specificities that recognize those peptides. More broadly, stimulator cells, and more specifically dendritic cells, can be incubated in the presence of the whole Mhsp65 protein. As a further alternative, stimulator cells, and more specifically dendritic cells, can be transduced or transfected with RNA or DNA comprising the polynucleotide sequence of Mhsp65. Under these alternative conditions, peptide epitopes that are naturally cleaved out of the Mhsp65 protein, and which are generated in addition to peptide epitopes of SEQ ID NO: 2, 3, 4, 7, 17 and 31 can associate with an appropriate class I MHC molecule, which may or may not include HLA IA.

In specific embodiments, the methods of the present invention include a method that comprises contacting a CTL precursor lymphocyte with an antigen presenting cell that has bound an immunogenic peptide selected from a group comprising the peptides disclosed according to the invention.

In specific embodiments, the methods of the present invention include a method for inducing a CTL response *in vitro* that is specific for an infectious microbe HLA IA and Mhsp65, or proteins highly homologous thereto, whereby the method comprises contacting a CTL precursor lymphocyte with an antigen presenting cell that has exogenously acquired an immunogenic oligopeptide or polypeptide that comprises one or more of the peptides disclosed according to the invention.

An additional embodiment relates to a method of inducing a CTL response *in vitro* that is specific for an infectious microbe expressing the Mhsp65 protein, or a protein highly homologous thereto, whereby the method comprises contacting a CTL precursor lymphocyte with an
5 antigen presenting cell that is expressing a polynucleotide encoding a polypeptide comprising an epitope differing by not more than 2 amino acid residues from SEQ ID NO: 2, 3, 4, 7, 17 and 31, especially 4.

A yet additional embodiment of the present invention is directed to a method of inducing a CTL response *in vitro* that is specific for an
10 infectious microbe expressing HLA IA and Mhsp65, or a protein highly homologous thereto. Such a method comprises contacting a CTL precursor lymphocyte with an antigen presenting cell that is expressing a polynucleotide coding for at least one CTL epitope of the invention, the polynucleotide is operably linked to a promoter and wherein the
15 polynucleotide sequence does not include the entire Mhsp65 gene.

A variety of techniques exist for assaying the activity of CTL. These techniques include the labeling of target cells with radionuclides such as $\text{Na}_2^{51}\text{CrO}_4$ or ^3H -thymidine, and measuring the release or retention of the radionuclides from the target cells as an index of cell death. Such
20 assays are well-known in the art and their selection is left to those skilled in the art. Alternatively, CTLs are known to release a variety of cytokines when they are stimulated by an appropriate target cell. Non-limiting examples of such cytokines include IFN- γ , TNF α , and GM-CSF. Assays for these cytokines are well known in the art, and their selection is left to the
25 skilled artisan. Methodology for measuring both target cell death and cytokine release as a measure of CTL reactivity are given in (Coligan, J. E. et al, Current Protocols in Immunology, 1999, John Wiley & Sons, Inc., New York).

After expansion of the antigen-specific CTL, the cells can then be adoptively transferred back into the patient, where they will destroy their specific target cell. The utility of such adoptive transfer is demonstrated in (North, R. J. et al., Infect.Immun., 67:2010-2012, (1999); Riddell, S. R. et al., Science, 257:238-241, (1992)). In determining the amount of cells to reinfuse, the skilled physician will be guided by the total number of cells available, the activity of the CTL as measured *in vitro*, and the condition of the patient. Preferably, however, about 1×10^6 to about 1×10^{12} , more preferably about 1×10^8 to about 1×10^{11} , and even more preferably, about 1×10^9 to about 1×10^{10} peptide-specific CTL are infused. Methodology for re-infusing the T cells into a patient are well known and exemplified in U.S. Patent No. 4,844,893 to Honski, et al., and U.S. Patent No. 4,690,915 to Rosenberg.

The peptide-specific CTL can be purified from the stimulator cells prior to infusion into the patient. For example, monoclonal antibodies directed towards the cell surface protein CD8, present on CTL, can be used in conjunction with a variety of isolation techniques such as antibody panning, flow cytometric sorting, and magnetic bead separation to purify the peptide-specific CTL away from any remaining non-peptide specific lymphocytes or from the stimulator cells. These methods are well-known in the art, and are their selection is left to the skilled artisan.

Another embodiment of the present invention relates to a method of treating a patient infected with a microbe expressing complexes of HLA-IA and Mhsp65, or a protein homologous, preferably highly homologous, thereto, whereby CTLs produced *in vitro* according to the present invention are administered in an amount sufficient to destroy the microbial cells through direct lysis or to effect the destruction of the microbial cells indirectly through the elaboration of cytokines.

Another embodiment of the present invention is directed to a method of treating a patient infected with a microbe expressing any class I MHC molecule and a gene encoding a protein homologous to Mhsp65, whereby the CTLs are produced *in vitro* and are specific for the Mhsp65 gene and are administered in an amount sufficient to destroy the infectious microbes through direct lysis or to effect the destruction of the infectious microbes indirectly through the elaboration of cytokines.

The present invention also relates to a method for inducing a CTL response in a subject comprising administering an immunogen comprising a peptide having an amino acid sequence selected from the group consisting of (a) SEQ ID NO: 2, 3, 7, 17 and 31; (b) a sequence differing from (a) by at least one but no more than 2 amino acids; (c) SEQ ID NO: 4; (d) a sequence differing from (c) by at least one but no more than 2 amino acids at least one CTL epitope, and wherein said immunogen is other than a bacterial heat shock protein 65 and wherein said peptide is other than a peptide contained in human heat shock protein 65.

In another embodiment, the present invention is directed to a method for inducing a CTL response in a subject, comprising administering to a patient suffering from a microbial infection or at risk of such infection a polynucleotide encoding an immunogen recited above, including active fragments thereof, in an amount sufficient to induce a CTL response to infectious microbes. Such polynucleotide may be contained within a plasmid or within a viral vector.

The present invention also relates to a method for inducing a CTL response in a subject, comprising administering to a patient suffering from a microbial infection or at risk of such infection a polynucleotide encoding a minigene an immunogen recited above in an amount sufficient to induce a CTL response to infectious microbes.

The *ex vivo* generated CTL can also be used to identify and isolate the T cell receptor molecules specific for the peptide. The genes encoding the alpha and beta chains of the T cell receptor can be cloned into an expression vector system and transferred and expressed in naïve T cells
5 from peripheral blood, T cells from lymph nodes, or T lymphocyte progenitor cells from bone marrow. These T cells, which would then be expressing a peptide-specific T cell receptor, would then have anti-microbial activity.

10 Alternatively, the immunogenic peptides disclosed herein, as well as functionally similar homologs thereof, may be used to screen a sample for the presence of CTLs that specifically recognize the corresponding epitopes. The lymphocytes to be screened in this assay will normally be obtained from the peripheral blood, but could be obtained from other
15 tissues. The peptides of the present invention may then be used as a diagnostic tool to evaluate the efficacy of the immunotherapeutic treatments disclosed herein. Thus, the *in vitro* generation of CTL as described above would be used to determine if patients are likely to respond to the peptide *in vivo*. Similarly, the *in vitro* generation of CTL
20 could be done with samples of lymphocytes obtained from the patient before and after treatment with the peptides. Successful generation of CTL *in vivo* should then be recognized by a correspondingly easier ability to generate peptide-specific CTL *in vitro* from lymphocytes obtained following treatment in comparison to those obtained before treatment.

25 As indicated above, a vaccine in accordance with the present invention may include one or more of the hereinabove described polypeptides or active fragments thereof, or a composition, or pool, of immunogenic peptides disclosed herein. When employing more than one

polypeptide or active fragment, such as two or more polypeptides and/or active fragments may be used as a physical mixture or as a fusion of two or more polypeptides or active fragments. The fusion fragment or fusion polypeptide may be produced, for example, by recombinant techniques or
5 by the use of appropriate linkers for fusing previously prepared polypeptides or active fragments.

The immunogenic molecules of the invention, including vaccine compositions, may be utilized according to the present invention for purposes of preventing, suppressing or treating infections causing the
10 expression of the immunogenic peptides disclosed herein, such as where the antigen is being expressed by microbial cells. As used in accordance with the present invention, the term "prevention" relates to a process of prophylaxis in which an animal, especially a mammal, and most especially a human, is exposed to an immunogen of the present invention prior to
15 the induction or onset of the infectious process. Alternatively, the immunogen could be administered to the general population as is frequently done for infectious diseases like those disclosed herein. The term "treatment" is often utilized to mean the clinical application of agents to combat an already existing conditions whose clinical
20 presentation has already been realized in a patient. This would occur where an individual has already been diagnosed as having an infection.

The peptides and polypeptides of the invention may also be delivered with an adjuvant. Adjuvants include, but are not limited to complete or incomplete Freund's adjuvant, Montanide ISA-51, aluminum
25 phosphate, aluminum hydroxide, alum, and saponin. Adjuvant effects can also be obtained by injecting a variety of cytokines along with the immunogens of the invention. These cytokines include, but are not limited to IL-1, IL-2, IL-7, IL-12, and GM-CSF.

The peptides and polypeptides of the invention can also be added to professional antigen presenting cells such as dendritic cells that have been prepared ex vivo. For example, the dendritic cells could be prepared from CD34 positive stem cells from the bone marrow, or they could be prepared from CD14 positive monocytes obtained from the peripheral blood. The dendritic cells are generated ex vivo using cytokines such as GM-CSF, IL-3, IL-4, TNF, and SCF. The cultured DC are then pulsed with peptides at various concentrations using standard methods that are well known in the art. The peptide-pulsed dendritic cells can then be administered either intravenously, subcutaneously, or intradermally, and the immunization may also include cytokines such as IL-2 or IL-12.

A specific embodiment of the present invention relates to a method for inducing a CTL response in a subject, wherein the immunogen is in the form of one or more peptides. The method comprises administering to subjects that express HLA IA, at least one CTL epitope, wherein said epitope or epitopes are selected from a group comprising the peptides disclosed according to the invention, in an amount sufficient to induce a CTL response to an infectious microbe, especially those disclosed herein, expressing HLA IA and a protein homologous to mycobacterial heat shock protein 65.

A further embodiment of the present invention relates to a method for inducing a CTL response in a subject, wherein the immunogen is in the form of one or more oligopeptides or polypeptides. The method comprises administering to subjects that express HLA IA at least one CTL-inducing epitope, wherein said epitope or epitopes are selected from a group comprising the peptides disclosed according to the invention, especially epitopes differing by no more than two amino acids from SEQ ID NO: 2, 3, 4, 7, 17 and 31, and are contained within oligopeptides or polypeptides that do not comprise the entire Mhsp65 or Huhsp65, in an

amount sufficient to induce a CTL response to infectious microb s, especially those disclosed herein, most especially *M. tuberculosis*, expressing HLA IA and a protein homologous to mycobacterial heat shock protein 65 (Mhsp65).

5 A still further embodiment of the present invention relates to a method for inducing a CTL response in a subject, wherein the immunogen is in the form of a polynucleotide. The method comprises administering to subjects that express HLA IA and at least one CTL-inducing epitope, wherein said epitope or epitopes are selected from a group comprising the
10 epitopic peptides disclosed according to the invention, and are coded within a polynucleotide sequence that does not comprise the entire Mhsp65 gene or Huhsp65 gene, in an amount sufficient to induce a CTL response to infectious microbes, especially those disclosed herein (see Tables 3 and 4), most especially *M. tuberculosis*, expressing HLA IA and
15 a protein homologous to mycobacterial heat shock protein 65 (Mhsp65).

 In carrying out the procedures of the present invention it is of course to be understood that reference to particular buffers, media, reagents, cells, culture conditions and the like are not intended to be limiting, but are to be read so as to include all related materials that one
20 of ordinary skill in the art would recognize as being of interest or value in the particular context in which that discussion is presented. For example, it is often possible to substitute one buffer system or culture medium for another and still achieve similar, if not identical, results. Those of skill in the art will have sufficient knowledge of such systems and methodologies
25 so as to be able, without undue experimentation, to make such substitutions as will optimally serve their purposes in using the methods and procedures disclosed herein.

 The present invention will now be further described by way of the
30 following non-limiting examples. In applying the disclosure of th s

examples, it should be kept clearly in mind that other and different embodiments of the methods disclosed according to the present invention will no doubt suggest themselves to those of skill in the relevant art.

5

GENERAL METHODS USED

For purposes of the disclosure herein, the following abbreviations are used: Ag, antigen; Ab, antibody; CTL, cytotoxic T lymphocyte; HLA, human leukocytes antigen; hsp, heat shock protein; bacillus Calmette-Guérin; MHC, major histocompatibility complex; IFA, incomplete Freund's adjuvant; IFN, interferon; p, plasmid; Th, T helper; TNF, tumor necrosis factor.

15 Plasmids

Mycobacterium bovis bacille Calmette-Guérin hsp 65 cDNA was excised from plasmid pRIB1300 (kindly provided by Dr. R. v d Zee, Utrecht University, The Netherlands). It was cloned in the multiple cloning site of plasmid pcDNA3 (Invitrogene, The Netherlands). The identity and the orientation of the gene in the resulting plasmid (p3M.65) was tested by restriction mapping. The expression was tested by transient transfection in COS-7 cells followed by western blot and immune detection by an anti Mycobacterial hsp65 specific monoclonal antibody DC-16 (kindly provided by Dr. Joraj Ivanyi, London, UK). The resulting band confirmed to the size of Mhsp 65 as judged by comparison to molecular weight marker and to the recombinant Mhsp 65 protein. Details about these procedure are to be published elsewhere (J. Charo et al., manuscript in preparation).

30 Peptide selection and synthesis

Candidate HLA-A*0201 binding peptides from hsp65 were selected using the MOTIFS software (52) and by the algorithm by K.C. Parker (see Parker et al, Sequence motifs important for peptide binding to the human MHC Class I molecule, HLA-A2, *J. Immunol.* **149**, 3580 (1992) and
5 Parker et al, Peptide binding to Class I MHC molecules: Implications for antigenic peptide prediction, *Immunol. Res.* **14**, 34 (1995). Positive scores were given for each potential anchor residue found in the peptide, and negative scores for individual anchor and inhibitor residues. All peptides with predicted scores of at least 44 were selected as potential
10 binders.

Peptides were made on an Abimed 422 synthesizer (Langerfeld, Germany) using TentagelS AC resins (Rapp, Tübingen, Germany) in combination with Fmoc chemistry (53). The purity of the peptides was
15 checked on reversed phase HPLC and was shown to be routinely over 75%. As the standard in the peptide binding assay the peptide HBV core p47-56 was used in which position 52 was substituted with cysteine (FLPSDCFPSV, SEQ ID NO: 57). Labeling of the cysteine in the peptides was performed with 4-(iodoacetamido) fluorescein (Fluka Chemie AG,
20 Buchs) at pH 8 in a mixture of 250 µl Sodium phosphate buffer 0.15 M and 150 µl acetonitrile (54). Peptides were purified on HPLC before use.

HLA-A*0201, β 2m and HLA-A*0201 Peptide binding assay

Recombinant HLA-A*0201 was over-expressed in *E. coli*, purified
25 as described (55) and dissolved in 8 M urea. The integrity of the protein was confirmed by Maldi-Tof mass spectrometry using insulin as an internal reference. Human β 2m was purchased from Sigma (St. Louis, Mo.) and dissolved in H₂O. Heavy chain (50 mM) stock solutions were stored at -20°C until use.

30

HLA-A*0201 was titrated in the presence of 100 fmol standard peptide to determine the HLA concentration necessary to bind 20-50% of the total fluorescent signal as described (56).

5 Mice

HLA-A*0201/Kb transgenic mice (kindly provided by Dr. L. Sherman, Scripps laboratories, San Diego, CA) used in this study have been described (49). Mice were propagated and held in our SPF environment in the MTC animal house at the Karolinska Institute. These mice express a chimeric MHC class I molecule in which the $\alpha 1$ and $\alpha 2$ domains are of the HLA-A*0201 molecule while the $\alpha 3$, transmembrane and cytoplasmic domains are of the mouse H2 Kb molecule. This construction permits the binding of mouse CD8 molecule on the T cell to interact with the $\alpha 3$ domain of the chimeric molecule. The surface expression of the HLA-A*0201/Kb was confirmed using HLA-A*0201 specific FITC-conjugated monoclonal antibody (One Lambda, CA) and assessed by flow cytometry using FACSCAN (Becton Dickinson & Co., Mountain View, CA).

20 Immunization

Eight to fourteen weeks old female or male mice were used in these experiments. Each mouse received either 20 μ g of p3M.65 plasmid i. m. and an adjuvant (J. Charo et.al. manuscript in preparation) in PBS or 50 μ g peptide plus 1% BSA in PBS emulsified with equal volume of IFA as described earlier (57, 58) (Sigma, St. Louis, MI).

Target Cell lines

Jurkat A*0201/Kb, a human T cell leukemia HLA-A*0201 negative cells stably transfected with the HLA-A*0201/Kb chimeric gene (kindly provided by Dr. W. M. Kast, Loyola university, Maywood, IL). The T2 is a

TAP 1/2 deficient and HLA-A2⁺ cell line. JY is an HLA-A2⁺ B-lymphoblastoid cell line.

The generation of specific CTL lines and clones

5 Peptide specific mouse CTL lines were prepared in 12 well plates as follows. Splenocytes, from immunized or control non-immunized mice, were plated at 6×10^6 cell/well and co-cultured with three million peptide pulsed (5 μ g) syngeneic splenocytes. After 6-8 days they were assayed for cytotoxicity as indicated below.

10

 Human CTL lines were generated by using monocyte derived dendritic cells, pulsed with peptide (50 μ g/ml for 3 hours in the presence of 3 μ g/ml β 2m (Sigma), as stimulator cells for a responder population of purified CD8⁺ T cells. The culture medium was IMDM (Gibco)
15 supplemented with 10 % human pooled serum. After seven days of culture with recombinant IL7 (20 ng/ml) and IL12 (100 pg/ml) (Genzyme, NV, Leuven, Belgium), IL2 (Cetus) was added at a final concentration of 25 CU/ml. Cells were restimulated weekly using autologous peptide pulsed monocytes. At the same time cells were checked by FACS
20 analysis (N.V. Becton Dickinson S.A., Belgium) for the % of CD8⁺ and CD4⁺ cells. If the % CD8 was lower than 90%, the CD4⁺ cells were depleted using MACS (Miltenyi Biotec GmbH) beads.

Cytotoxicity assays

25 Cell mediated cytotoxicity was measured by a ⁵¹Cr release assay. One million target cells were incubated at 37 C in the presence of 200 μ Ci of Sodium ⁵¹Cr Chromate (Amersham, UK) for 1 hour, washed three times and re-suspended in complete medium at 10^5 cells/ml in the presence or absence of 10 μ g of the relevant peptide. The test was
30 performed by incubating 5×10^3 target cells at different effector to target ratio in triplicate wells at a final volume of 200 μ l in v-bottomed 96 well

plates. They were incubated for 4 hours at 37°C after which supernatants were harvested and used to determine specific lysis using the following equation: percent specific release = $100 \times (\text{experimental release} - \text{spontaneous release}) / (\text{maximum release} - \text{spontaneous release})$.

5

Proliferation Test

Splenocytes were harvested from immunized mice. A single cell suspension was prepared and cells were re-suspended at 3×10^6 cells per ml in IMDM supplemented with 10% FBS and L-Glu and antibiotics.

- 10 Peptides were added at 5 µg/ml, Mhsp65 and PPD at 10 g/ml concentrations. Cultures were incubated for 5 days at 37°C in 7.5% CO₂. One mCi of tritium labeled thymidine was added to each well of U shaped bottom 96 well plates. Cells were further incubated for 18 hours in the same conditions as above and harvested. The amount of incorporated
- 15 tritium labeled thymidine was measured using Beta Plate reader (Wallac, Turku, Finland). Test samples were set up in triplicates and the SDs were ≤10%.

Cytokine assays

Mixed CTL and monocytes cultures were prepared by mixing 1×10^6 CTLs plus 1×10^5 monocytes per ml. Supernatants were collected after 72 hours of culture and were tested for the presence of IFN- γ using commercially available matched antibody pairs (Mabtech, Stockholm) according to the manufacturers procedures. Alternatively, 18 hour-supernatants were added to WEHI cells for the determination of TNF- α concentrations. IFN- γ ELISPOT was performed in 96 microwell plates (Millipor SA, Molsheim, France) which were coated with 10 μ g/ml of capturing antibody R4-6A2 (hybridoma purchased from ATCC, VA) and then blocked with AIM-V 10 % FCS (Life technology, Paisly, Scotland) for 2 hours in 37°C. Irradiated splenocytes (2.5×10^5) were added as APCs to each well. 500 cells from Clone D1 were then added to the splenocytes together with the peptide at different concentrations. Cells were incubated for 16 hours, with or without blocking with the HLA class I specific antibody W6/32 (hybridoma purchased from ATCC, VA), in 37°C and 10% CO₂ atmosphere. The plates were washed 6x with PBS + 0.05% Tween followed by the addition of the biotin labeled rat Anti-Mouse IFN- γ Ab (Pharmingen, CA) and incubated for 2 hours at room temperature. After 6 further washes with PBS + 0.05% Tween Streptavidin-ALP was added and the plates were incubated for one hour at room temperature. Finally spots were developed with substrate (NBT/BCIP combo, Life technology, Paisly, Scotland). The spot counting was performed using a stereo microscope.

25

Proteasome digestion assay

20S proteasomes were purified from RMA cells and HeLa cells as described (59). Peptides (26-mers were incubated with 1 μ g of purified proteasome at 37°C for 30 minutes, 2 and 8 hours in 300 μ L of proteasome digestion buffer (20mM HEPES pH-7.8, 2mM MgAc₂, 1mM

30

DTT). Addition of 10 μ L of acetic acid stopped the digestion. Before analysis by MS, peptide digestions were kept at -70°C .

Mass spectrometry

5 Electrospray ionization mass spectrometry was performed on a hybrid quadrupole time-of-flight mass spectrometer, a Q-TOF (Micromass, Manchester, UK), equipped with an on-line nanoelectrospray interface (capillary tip 20 μm internal diameter \times 90 μm outer diameter) with an approximate flow rate of 250 nl/min . This flow was obtained by splitting
10 of the 0.4 ml/min flow of a conventional high pressure gradient system 1 to 1000, using an Accurate flow splitter (LC Packings, Amsterdam, The Netherlands). Injections were done with a dedicated micro/nano HPLC autosampler, the FAMOS (LC Packings, Amsterdam, The Netherlands), equipped with two extra valves for phase system switching experiments.
15 Digestion solutions were diluted five times in water/methanol/acetic acid 95/5/1 v/v/v, and 1 μL was trapped on the precolumn (MCA-300-05-C8; LC Packings, Amsterdam) in water/methanol/acetic acid 95/5/1 v/v/v. Washing of the precolumn was done for three minutes to remove the buffers present in the digests. Subsequently the trapped analytes were
20 eluted with a steep gradient going from 70% B to 90% B in 10 minutes, with a flow of 250 nl/min . (A: water/methanol/acetic acid 95/5/1; B: water/methanol/acetic acid 10/90/1). This low elution flow rate allows for a few additional MS/MS experiments if necessary during the same elution. Mass spectra were recorded from mass 50-2000 Da every second with a
25 resolution of 5000 FWHM. The resolution allows direct determination of the monoisotopic mass, also from multiple charged ions. In MS/MS mode ions were selected with a window of 2 Da with the first quadrupole and fragments were collected with high efficiency with the orthogonal time-of-flight mass spectrometer. The collision gas applied was argon (4×10^{-5} mbar), and the collision voltage appr. 30 V. The peaks in the mass
30

spectra were searched in the digested 26-mer peptides using the Biolynx/proteins software supplied with the mass spectrometer.

Identification of peptides from mycobacterial hsp 65 that bind to HLA-A*0201.

The Mhsp 65 amino acid sequence was analyzed for the presence of HLA-A*0201 consensus binding motifs, as previously described (52), and the 54 highest scoring peptides were synthesized. Twenty-three were nonamer, 18 decamer and 13 were eleven-mer peptides. The amino acid sequences and the position of these peptides are shown in Table I.

To determine the binding affinity of the Mhsp 65 peptides to HLA-A*0201, peptides were analyzed for their ability to compete with a standard peptide (HBV core₄₇₋₅₆) in a fluometric assay (54, 56). While the majority of these peptides (n=25) were high affinity binders with an IC₅₀ of ≤ 0.75 μ M, 18 of them were intermediate affinity binders (IC₅₀ 1.4-14 μ M) and the rest (n=11) were low affinity or non-binders (IC₅₀ > 20 μ M). As the majority of TCR epitopes are high- or intermediate- affinity MHC class-I binders, and as low to non-binding peptides were recently shown to be poorly processed naturally and non immunogenic (51, 60), we restricted our further studies to the 43 high- and intermediate- binding, potential CTL epitope encoding peptides.

EXAMPLE 1

Induction of HLA-A*0201 specific CTL responses by DNA immunization and establishment of Mhsp 65 peptide specific T cell lines

A2/Kb transgenic mice were immunized by intra-muscular injection of a plasmid containing the full length M. hsp65 gene (p3M.65) co-injected with an adjuvant, which enhances DNA immunization. Two

weeks after the primary or the secondary immunization, spleen cells from immunized mice were re-stimulated in vitro with syngeneic splenocytes pulsed with 5 µg/ml of each of the 43 potential CTL epitopes. Five to seven days later, cells were tested for peptide specific, HLA-A*0201 restricted cytotoxicity, using as target cells the human T cell line Jurkat transfected with an HLA-A2/Kb (Jk-A2/Kb) construct with or without peptide. Cytotoxicity was considered specific when the percent of ⁵¹Cr release was more than 15 percent and the cytotoxicity towards the relevant peptide coated targets was more than twice that seen for negative control targets. Based on these criteria six HLA-A*0201 restricted CTL epitopes were identified: peptides Mhsp65(9₄₁₆), Mhsp65(9₃₆₂), Mhsp65(9₃₆₉), Mhsp65(10₉₇), Mhsp65(11₅₀₀) and Mhsp65(9₂₁) which all triggered a moderate to strong CTL response (Fig. 1a-d).

15

Peptide specific CTL lines were then tested for their ability to recognize and kill the human T2 cell line, an HLA-A*0201 target that does not express the mouse CD8 binding region of MHC class I. T2 cells were strongly lysed by the Mhsp65(9₃₆₉) specific CTL line when pulsed with the cognate peptide, while no specific response could be detected against pulsed T2 cells with the Mhsp65(9₄₁₆), Mhsp65(9₃₆₂) or Mhsp65(10₉₇) specific CTL lines (Figure 2). These results strongly suggest that peptide Mhsp65(9₃₆₉) induces high-affinity CTL which can lyse target cells even without the additional binding affinity conferred by CD8-MHC class I interaction (61), while CTL induced against other Mhsp65 epitopes were dependent on CD8. Since DNA immunization largely results in antigen presentation via the endogenous MHC class I processing pathway, it is also likely that these Mhsp65 epitopes are "naturally" processed (see also below).

30

Due to the high homology of hsp65 among organisms we checked for the homology between the defined hsp65 epitopes and the corresponding sequences from other pathogens. Of particular interest, epitope Mhsp65(9₃₆₉) was identical in a large number of pathogens. When
 5 all public databases (Non-Redundant GenBank CDS translations + PDB + SwissProt + PIR + PRF Peptide Sequence Databases) were BLAST searched there were more than 500 sequences which were identical to or shared a high homology to this peptide. Homologous sequences were here defined according to the criteria of the BLASTp
 10 search program at NCBI (www.ncbi.nlm.nih.gov/blast/). These aligned homologous sequences are given in Table 4 (a good indicator for potential substitutions), together with their protein and species of origin as well as starting and ending amino acid numbers. The peptides covering most of these homologous sequences were tested by the inventors for cross
 15 reactivity with a T cell line specific for the Mhsp65(9₃₆₉) epitope. As can be seen in Table 3, the Mhsp65(9₃₆₉) reactive T cell line was found to recognize several of these peptide homologues derived from human pathogenic microorganisms

20 Peptides Mhsp65(9₄₁₆), Mhsp65(9₃₈₂), Mhsp65(10₉₇),
 Mhsp65(11₅₀₀) and Mhsp65(9₂₁) were identical (see Table 4) to the corresponding hsp65 sequences from only a limited number (n<10) of these pathogens. Moreover, an analogue to Mhsp65(9₃₆₉) in which isoleucine at position 9 was substituted with leucine, which is the
 25 corresponding sequence from hsp65 in some pathogenic bacteria such as Porphyromonas Gingivalis (PG), was also able to induce specific CTL from the splenocytes of Mhsp65 pDNA immunized mice. This CTL line lysed the JK-A2/Kb cells pulsed with the analogue PGhsp65(9₃₆₉) but not the unpulsed or HIV peptide pulsed cells (Fig. 3). Thus, and because of its
 30 strong immunogenicity in the HLA A2/Kb model, the Mhsp65(9₃₆₉) epitope was chosen for further analysis.

EXAMPLE 2

**The induction of HLA-A*0201 specific cytotoxic, proliferative and
5 cytokine responses by peptide immunization and establishment of
Mhsp65(9₃₆₉) specific CTL clones**

To test the ability of Mhsp65(9₃₆₉) to induce HLA-A*0201
restricted CTL we immunized A2/Kb transgenic mice subcutaneously with
10 50 µg of the Mhsp65(9₃₆₉) peptide or with the control influenza MP
derived peptide emulsified in IFA. Ten to fifteen days later splenocytes
from these mice were tested for their ability to kill Jk-A2/Kb target cells
which were pulsed or unpulsed with Mhsp65(9₃₆₉), or the corresponding
homologous epitope from human hsp65 (Huhsp65(9₃₉₆)), which is
15 identical to the corresponding mouse sequence, or irrelevant control
peptides. A significant killing of Mhsp65(9₃₆₉) pulsed targets as compared
to control targets was observed. No killing of target pulsed with the
homologous human epitope Huhsp65(9₃₉₆) could be detected, ruling out
the possibility of inducing a cross reactive immune response to human
20 hsp65 upon immunization with the Mhsp65(9₃₆₉) (Fig. 4a).

A strong proliferative response to the Mhsp65(9₃₆₉) epitope, as well
as to recombinant Mhsp65 protein and PPD could be detected in
splenocytes from mice immunized with this epitope but not from mice
25 immunized with the A2 binding peptide from the influenza MP (Fig. 4b),
suggesting that the Mhsp65(9₃₆₉) epitope is naturally processed.

Three specific CTL clones were obtained from this CTL line by
limiting dilution. Clones B5, C2 and D1 all killed Mhsp65(9₃₆₉) pulsed
30 target cells (Fig. 5a). Two of these clones (C2 and D1) were also able to
produce IFN-γ in response to peptide specific stimulation as analyzed by

IFN- γ specific ELIspot assay (data not shown). Cells from clone D1 responded specifically to Mhsp65(9₃₆₉) and to PGhsp65(9₃₆₉) by producing IFN- γ , while the homologous Huhsp65(9₃₉₆) peptide or a control HIV peptide could not induce such a response (Fig. 5b). This response was
5 HLA class I restricted as blocking with class I specific antibody reduced the response to the background level (Fig. 5c).

EXAMPLE 3

10 **Proteasome-mediated processing of Mhsp65 and Huhsp65 peptides.**

To investigate the processing of the Mhsp65(9₃₆₉), an in vitro proteasome digestion analysis was performed. For this purpose three 26-mer synthetic peptides were used as substrates for proteasome digestion:
15 the first 26-mer peptide harbored the Mhsp65(9₃₆₉); the second 26-mer contained the human Huhsp65(9₃₉₆) homologue; and the third 26-mer peptide harbored another Mhsp65 9-mer sequence that bound with high affinity to HLA-A*0201 (Table1; peptide 9₅₀₉), but that was not recognized by CTL (Fig. 1a). In each case the 9-mer sequence was
20 flanked by the naturally surrounding amino acids. For these experiments 20S proteasomes purified from the murine cell line RMA and the human cell line HeLa were used. After digestion at different time points the resulting peptide fragments were identified by Mass spectrometry as shown in Table 2. Both human and mouse proteasomes generate almost
25 identical digestion products from the Mhsp65(9₃₆₉) containing 26-mer, indicating that processing of this epitope in both transgenic mouse and human is similar. Figure 6 (panels A and C) shows the generation of the relevant epitope containing peptide fragments from the Mhsp65 sequence in time. The C-terminus of the epitope is used as a dominant proteasome
30 cleavage site whereas three different N-terminal cleavage sites are used resulting in three epitope-containing fragments that can all be relevant

epitope precursors. Comparison of proteasome-mediated processing of the Huhsp65(9₃₉₆) human homologue sequence reveals that no potential epitope-containing fragment could be detected (Fig.6, panels B and D). Only a long 18-mer precursor could be detected that decreased by time, suggesting further cleavage of this fragment into irrelevant small fragments for CTL recognition. Similarly, the Mhsp65(9₅₀₉) containing 26-mer yielded no potential epitope-containing fragments (Table 2).

These experiments show that the Mhsp65(9₃₆₉) epitope can be efficiently processed from its natural amino acid sequence, whereas the human homologue peptide as well as another Mhsp65 peptide that was not recognized by CTL, are not generated, thereby precluding induction of MHC class I restricted responses to these peptides.

15

EXAMPLE 4

The induction of Mhsp65(9₃₆₉) specific human CTL

Two essential questions relating to vaccine development are whether Mhsp65(9₃₆₉) specific T cells are present in the human T cell repertoire, and whether such T cells are able to recognize mycobacterium-infected macrophages. In order to address these issues, PBL from a HLA-A*0201+ donor were stimulated by Mhsp65(9₃₆₉) loaded, in vitro cultured mature autologous DC. Cells were repeatedly depleted of CD4⁺ T cells, resulting in the production of a T line with >90% CD8⁺ T cells as determined by FACs analysis (data not shown). After three restimulations, this CTL line lysed JY target cells pulsed with peptide Mhsp65(9₃₆₉) but not with a control peptide (Fig. 7a). The same CTL line also produced TNF- α in response to peptides Mhsp65(9₃₆₉) and PGhsp65(9₃₆₉) (Fig. 7b). Importantly, this CTL line was also able to produce TNF- α and IFN- γ in response to BCG infected but not to non-

infected monocytes (Fig. 7 c and d), showing that the Mhsp65(9₃₆₉) epitope is processed and presented by mycobacterium infected human macrophages.

5

Table 1. List of HLA-A*0201 binding peptides from hsp-65 and their binding affinities

Peptide	Sequence	SEQ ID NO	Position	IC ₅₀ (μM)*
Mhsp65(9 ₅₀₉)	GLFLTTEAV	1	509-517	0.05
Mhsp65(9 ₄₁₆)	TLLQAAPTL	2	416-424	0.05
Mhsp65(9 ₃₆₂)	KLQERLAKL	3	362-370	0.10
Mhsp65(9 ₃₆₉)	KLAGGVAVI	4	369-377	0.12
Mhsp65(9 ₂₃₃)	PLLEKVIGA	5	233-241	0.13
Mhsp65(11 ₃₆₆)	RLAKLAGGVAV	6	366-376	0.13
Mhsp65(10 ₉₇)	ALVREGLRNV	7	97-106	0.13
Mhsp65(10 ₄₈₅)	LLAAGVADPV	8	485-494	0.18
Mhsp65(9 ₃₆₆)	RLAKLAGGV	9	366-374	0.19
Mhsp65(9 ₂₈₆)	MLQDMAILT	10	286-294	0.29
Mhsp65(9 ₁₈)	GLNALADAV	11	18-26	0.30
Mhsp65(10 ₃₀₅)	LTLENADLSL	12	305-314	0.33
Mhsp65(10 ₂₁₂)	VLEDPYILLV	13	212-221	0.43
Mhsp65(9 ₂₁₂)	VLEDPYILL	14	212-220	0.43
Mhsp65(10 ₂₁₉)	LLVSSKVSTV	15	219-228	0.47
Mhsp65(11 ₃₁₃)	SLLGKARKVVV	16	313-323	0.53
Mhsp65(11 ₅₀₀)	ALQNAASIAGL	17	500-510	0.53

Mhsp65(10 ₂₁)	ALADAVKVTL	18	21-30	0.57
Mhsp65(9 ₃₁₃)	SLLGKARKV	19	313-321	0.57
Mhsp65(9 ₁₂₉)	TLLKGAKEV	20	129-137	0.57
Mhsp65(11 ₂₁₈)	ILLVSSKVSTV	21	218-228	0.58
Mhsp65(9 ₃₀₆)	TLENADLSL	22	306-314	0.60
Mhsp65(9 ₂₁₇)	YILLVSSKV	23	217-225	0.70
Mhsp65(9 ₃₀₄)	GLTLENADL	24	304-312	0.70
Mhsp65(11 ₁₈)	GLNALADAVKV	25	18-28	0.75
Mhsp65(11 ₃₀₄)	GLTLENADLSL	26	304-314	1.0
Mhsp65(10 ₅₀₉)	GLFLTTEAVV	27	509-518	1.0
Mhsp65(10 ₆₇)	KIGAELVKEV	28	67-76	1.2
Mhsp65(11 ₄₈₄)	DLAAGVADPV	29	484-494	1.4
Mhsp65(10 ₃₇₆)	VIKAGAATEV	30	376-385	1.7
Mhsp65(9 ₂₁)	ALADAVKVT	31	21-29	2.0
Mhsp65(10 ₂₉₈)	VISEEVGLTL	32	298-307	2.1
Mhsp65(9 ₁₄)	GLERGLNAL	33	14-22	2.1
Mhsp65(11 ₉₃)	VLAQALVREGL	34	93-103	3.2
Mhsp65(10 ₁₁₄)	GLKRGIEKAV	35	114-123	3.2
Mhsp65(9 ₂₆₄)	KIRGTFKSV	36	264-272	3.3
Mhsp65(11 ₄₁₇)	LLQAAPTLDEL	37	417-427	4.3
Mhsp65(10 ₁₅₈)	DLIAEAMDKV	38	158-167	5.0
Mhsp65(10 ₃₀₆)	TLENADLSLL	39	306-315	5.3
Mhsp65(9 ₁₁₈)	GIEKAVEKV	40	118-126	5.8
Mhsp65(11 ₄₅₆)	GLEPGVVAEKV	41	456-466	6.3

Mhsp65(10 ₂₁₈)	ILLVSSKVST	42	218-227	7.3
Mhsp65(10 ₂₉)	TLGPKGRNVV	43	29-38	14
Mhsp65(9 ₃₂₃)	VTKDETTIV	44	323-331	>20
Mhsp65(9 ₃₁₄)	LLGKARKVV	45	314-322	>20
Mhsp65(11 ₂₉)	TLGPKGRNVVL	46	29-39	>20
Mhsp65(11 ₄₄₇)	PLKQIAFNGL	47	447-457	>20
Mhsp65(11 ₃₁₁)	DLSLLGKARKV	48	311-321	>50
Mhsp65(10 ₃₁₃)	SLLGKARKVV	49	313-322	>50
Mhsp65(10 ₃₁₄)	LLGKARKVVV	50	314-323	>50
Mhsp65(10 ₂₃₀)	DLLPLLEKVI	51	230-239	>50
Mhsp65(9 ₂₄₄)	PLLIIAEDV	52	244-252	>50
Mhsp65(9 ₂₉)	TLGPKGRNV	53	29-37	>50
Mhsp65(9 ₆₀)	ELEDPYEKI	54	60-68	>50
Huhsp65(9 ₃₉₆)	KLSDGVAVL	55	396-404	6.5
PGhsp65(9 ₃₆₉)	KLAGGVAVL	56	369-377	0.12

* Binding affinity defined as the ability of the test peptide to inhibit 50% of the binding of a fluorescein labeled standard peptide to the recombinant HLA-A2 / β 2m complex as described in the experimental procedures.

Table 2: Overview of the peptide fragments generated by proteasome-mediated digestion

Sequence		Residue	Relative amount of peptide	
			RMA	HeLa
(A)				
5	EKLQERLAKLAGGVAVIKAGAATE	361-384	10.0	9.4
	EKLQERLAKLAGGVAVIKAGA	361-381	20.8	2.8
	EKLQERLAKLAGGVAVIKA	361-379	37.4	34.9
	EKLQERLAKLAGGVAVIK	361-378	3.7	4.6
	EKLQERLAKLAGGVAVI	361-377	3.0	2.9
10	EKLQERLAKLA	361-371	4.8	4.7
	EKLQERLAKL	361-370	11.9	23.3
	AKLAGGVAVIKAGAATEVE	368-386	1.1	2.4
	KLAGGVAVIKAGAATEVE	369-386	-	2.5
	AGGVAVIKAGAATEVE	371-386	2.8	5.7
15	GGVAVIKAGAATEVE	372-384	0.9	1.4
	KLQERLAKLAGGVAVIKAGAATE	362-384	0.8	0.7
	LAKLAGGVAVIKA	367-379	0.3	0.7
	AKLAGGVAVIK	368-378	0.4	1.1
	LAKLAGGVAVI	367-377	0.6	0.8
20	AKLAGGVAVI	368-377	0.4	0.2
	KLAGGVAVI	369-377	0.6	0.9

(B)

	KEKLNERLAKLSDGVAVLKV	386-406	6.6	2.2
	EKLNERLAKLSDGVAVL	387-404	3.5	2.2
	KEKLNERLAKLSDGVAV	386-403	-	1.0
5	KEKLNERLAKLSD	387-399	16.3	20.4
	KEKLNERLAKL	387-385	12.0	13.8
	AKLSDGVAVLKVGGTSDV	395-412	12.4	2.9
	SDGVAVLKVGGTSDV	386-412	10.6	12.8
	GVAVLKVGGTSDV	400-412	37.7	43.9
10	SDGVAVLKV	386-406	0.9	0.8

(C)

QNAASIAGLFLTTEAVVADKPEKEKA 502-527 100 100

26-mer synthetic peptides containing Mhsp65(9369)(A), Huhsp65(9396)(B) and a non recognized Mhsp65(9509)(C) sequence with their respective natural flanking amino acids were proteasome digested for 30 minutes. Purified 20S proteasomes from the murine cell line RMA and the human cell line HeLa are used. In bold are depicted the core HLA-A*0201 high affinity binding peptide sequences. The same fragments were found after longer digestion periods. The relative amount of peptide fragments generated by proteasome-mediated cleavage is given as percentage of the total amount of peptide fragments generated.

The Indicated sequences correspond, from top to bottom, to SEQ ID NO: 58 to 83, respectively.

Table 3

Summary of results demonstrating cross-reactivity between the mycobacterial hsp65 (Mhsp65) peptide KLAGGVAVI and peptides derived from homologous regions of hsp65 derived from other organisms.

Homologous Amino acid sequence *	Summary of results as tested by the ELISPOT assay ****	Examples of species from which the homologous heat shock protein was derived ****
KLAGGVAVI (Mhsp65(9 ₃₆₉)) **	+++	<i>Mycobacterium Tuberculosis</i>
KLSDGVAVL	-	<i>Homo Sapiens</i>
KLRNGVAVL	-	<i>Rickettsia tsutsugamushi</i>
KLSGGVAVI	+++	<i>Chlamydia, Trypanosoma, Helicobacter, Leishmania.</i>
KLTGGVAVI	+++	<i>Trichomonas vaginalis</i>
KLVGGVAVI	+++	<i>Synechoccus vulcanus</i>
KLSGGVAVL	++	<i>Rickettsia prowazekii, Cowdria ruminatum</i>

* Only the a.a. sequence is given, since the actual position in the molecule varies between different molecules containing this sequence.

** This peptide epitope is identical in a number of microorganisms (see Table 4), including: *Salmonella typhi*, *Vibrio cholerae*, *Yersinia enterocolitica*, *Bordetella pertussis*, *Staphylococcus aureus*.

*** Cross-reactivity is here defined arbitrarily, where +++ denotes a strong reactivity and - the absence of reactivity. This was tested by a CD8+ T cell line specific for the Mhsp65(9₃₆₉) peptide KLAGGVAVI, in an ELISPOT assay detecting the frequency of T cells responding by Interferon gamma production in response to the respective peptide as specified in column 1.

**** This column exemplifies species from which the homologous heat shock protein was derived.

Reference for ELISPOT assay;

1. Taguchi, T., J. R. McGhee, R. L. Coffman, K. W. Beagley, J. H. Eldridge, K. Takatsu, and H. Kiyono. 1990. Detection of individual mouse splenic T cells producing IFN-gamma and IL-5 using the enzyme-linked immunospot (ELISPOT) assay. *J Immunol Methods* 128:65.

Table 4 (continues through page 75)

GenBank BLASTp search (PDB+SwissProt+PIR+PRF)		Score (bits)	E Value	N	Start seq nr	Homologous Alignments	End seq nr
Sequences producing significant alignments:							
gb AAA23934.1	(M11294) a.k.a. mopA [Escherichia coli]	32	0.17	1	65	1 KLAGGVAVI	9
dbj BAA36516.2	(AB015985) chaperonin 60 [Paracoccus de...	32	0.17	1	371	73
sp Q9Z462 CH60_PARDE	60 KDA CHAPERONIN (PROTEIN CPN60) ...	32	0.17	1	371	379
sp O50323 CH61_SYNPU	60 KD CHAPERONIN 1 (PROTEIN CPN60) ...	32	0.17	1	369	377
sp P35469 CH61_RHIME	60 KDA CHAPERONIN A (PROTEIN CPN60)...	32	0.17	1	371	379
sp P95678 CH60_RHOCA	60 KD CHAPERONIN (PROTEIN CPN60) (...)	32	0.17	1	371	379
sp P48216 CH60_PSEPU	60 KD CHAPERONIN (PROTEIN CPN60) (...)	32	0.17	1	369	377
pir JN0509	heat shock protein groEL (clone Rhz A) - Rh...	32	0.17	1	371	379
sp P48211 CH60_CAUCR	60 KDA CHAPERONIN (PROTEIN CPN60) ...	32	0.17	1	370	378
pir JC2564	heat shock protein groEL - Zymomonas mobilis	32	0.17	1	371	379
dbj BAA03164.1	(D14078) Yersinia enterocolitica hsp60,...	32	0.17	1	371	379
gb AAD04240.1	(AF014831) 60 kDa heat shock protein [Ba...	32	0.17	1	300	308
gb AAA22898.1	(M98257) immunoreactive protein [Bartone...	32	0.17	1	371	379
sp P35635 CH60_BARBA	60 KDA CHAPERONIN (PROTEIN CPN60) ...	32	0.17	1	371	379
sp O68309 CH60_AERSA	60 KDA CHAPERONIN (PROTEIN CPN60) ...	32	0.17	1	371	379
gb AAF64162.1 AF239164_2	(AF239164) GroEL [Rhizobium le...	32	0.17	1	371	379
gb AAF33788.1 AF222061_1	(AF222061) heat shock protein ...	32	0.17	1	369	377
sp P28598 CH60_BACSU	60 KDA CHAPERONIN (PROTEIN CPN60) ...	32	0.17	1	369	377
pir B36917	heat shock protein GroEL - Agrobacterium tu...	32	0.17	1	371	379
emb CAA85784.1	(Z37730) putative chaperonine [Prochlor...	32	0.17	1	285	293
sp P29842 CH60_NEIGO	60 KDA CHAPERONIN (PROTEIN CPN60) ...	32	0.17	1	371	379
pir B43827	chaperonin groEL - Brucella abortus (strain...	32	0.17	1	369	377
gb AAC24232.1	(AF071193) 60 kDa heat shock protein [Ba...	32	0.17	1	288	296
emb CAB83768.1	(AL162753) chaperonin 60kD subunit [Nel...	32	0.17	1	371	379
pir S70667	chaperonin groEL - Caulobacter crescentus	32	0.17	1	369	377
sp P30779 CH60_AGRU	60 KDA CHAPERONIN (PROTEIN CPN60) ...	32	0.17	1	371	379
sp P42385 CH60_NEIME	60 KDA CHAPERONIN (PROTEIN CPN60) ...	32	0.17	1	371	379
gb AAF95805.1	(AE004332) chaperonin, 60 kd subunit [Vi...	32	0.17	1	371	379
gb AAF42301.1	(AE002545) chaperonin, 60 kDa [Neisseria...	32	0.17	1	371	379

sp P48215 CH60_NEIFL 60 KDA CHAPERONIN (PROTEIN CPN60) ...	32	0.17	1	371	379
sp P06806 CH62_MYCTU 60 KD CHAPERONIN 2 (PROTEIN CPN60-....	32	0.17	1	369	377
sp P45746 CH60_THETH 60 KDA CHAPERONIN (PROTEIN CPN60) ...	32	0.17	1	369	377
gb AAF43464.2 (AF240579) heat shock protein 60 [gadne...	32	0.17	1	369	377
dbj BAA25225.1 (AB008146) similar to GroEL protein [Kl...	32	0.17	1	371	379
emb CAB65482.1 (AJ250409) chaperonin-60 [Thermus therm...	32	0.17	1	369	377
sp O66206 CH60_SERMA 60 KDA CHAPERONIN (PROTEIN CPN60) ...	32	0.17	1	371	379
pir JC5771 chaperonin groEL-like protein - Weevil	32	0.17	1	371	379
gb AAA83441.1 (U29483) GroEL-like chaperonin [Thermus ...	32	0.17	1	369	377
sp O66198 CH60_ENTAE 60 KDA CHAPERONIN (PROTEIN CPN60) ...	32	0.17	1	371	379
gb AAD04241.1 (AF014832) 60 kDa heat shock protein [Ba...	32	0.17	1	334	342
sp O66220 CH60_ERWCA 60 KDA CHAPERONIN (PROTEIN CPN60) ...	32	0.17	1	371	379
gb AAD04243.1 (AF014834) 60 kDa heat shock protein [Ba...	32	0.17	1	312	320
sp P46224 CH60_PYRSA 60 KD CHAPERONIN (PROTEIN CPN60) (...	32	0.17	1	399	407
sp O66210 CH60_KLEOX 60 KDA CHAPERONIN (PROTEIN CPN60) ...	32	0.17	1	371	379
sp O66214 CH60_KLEON 60 KDA CHAPERONIN (PROTEIN CPN60) ...	32	0.17	1	371	379
gb AAD04239.1 (AF014830) 60 kDa heat shock protein [Ba...	32	0.17	1	288	296
gb AAF75593.1 AF214488_3 (AF214488) GroEL [Lactobacillu...	32	0.17	1	369	377
sp O66204 CH60_SERFI 60 KDA CHAPERONIN (PROTEIN CPN60) ...	32	0.17	1	371	379
sp O66212 CH60_KLEPL 60 KDA CHAPERONIN (PROTEIN CPN60) ...	32	0.17	1	371	379
sp P77829 CH61_BRAJA 60 KD CHAPERONIN 1 (PROTEIN CPN60 ...	32	0.17	1	371	379
gb AAA99670.2 (U15989) PTB65K [Mycobacterium avium sub...	32	0.17	1	410	418
sp O66194 CH60_ENTGE 60 KDA CHAPERONIN (PROTEIN CPN60) ...	32	0.17	1	371	379
gb AAD04242.1 (AF014833) 60 kDa heat shock protein [Ba...	32	0.17	1	334	342
sp Q9ZFE0 CH60_BURCE 60 KDA CHAPERONIN (PROTEIN CPN60) ...	32	0.17	1	371	379
gb AAA25354.1 (M14341) 65 kd antigen [Mycobacterium le...	32	0.17	1	416	424
sp P34939 CH60_RHILV 60 KD CHAPERONIN (PROTEIN CPN60) (...	32	0.17	1	371	379
pir A41468 60K heat shock protein htpB - Legionella pn...	32	0.17	1	372	380
sp P25967 CH60_BRUAB 60 KDA CHAPERONIN (PROTEIN CPN60) ...	32	0.17	1	371	379
sp P26194 CH60_LEGMI 60 KDA CHAPERONIN (PROTEIN CPN60) ...	32	0.17	1	370	378
sp P35861 CH62_BRAJA 60 KD CHAPERONIN 2 (PROTEIN CPN60 ...	32	0.17	1	371	379
pir I40342 heat shock protein - Brucella abortus >gi 1...	32	0.17	1	371	379

sp P35862 CH63_BRAJA_60_KD_CHAPERONIN_3 (PROTEIN CPN60) ...	32	0.17	1	371	379
sp P48219 CH60_YEREN_60_KDA_CHAPERONIN (PROTEIN CPN60) ...	32	0.17	1	371	379
pir S26423 heat shock protein 60 - Yersinia enterocoli...	32	0.17	1	371	379
pir A25902 65K antigen - Mycobacterium leprae	32	0.17	1	416	424
sp P35468 CH60_LEPIN_60_KD_CHAPERONIN (PROTEIN CPN60) (...)	32	0.17	1	370	378
sp P31293 CH60_CHRVI_60_KDA_CHAPERONIN (PROTEIN CPN60) ...	32	0.17	1	371	379
sp O78419 CH60_GUITH_60_KD_CHAPERONIN (PROTEIN CPN60) (...)	32	0.17	1	370	378
sp Q9ZFD8 CH60_BURVI_60_KDA_CHAPERONIN (PROTEIN CPN60) ...	32	0.17	1	371	379
sp P21239 RUB1_BRANA RUBISCO SUBUNIT BINDING-PROTEIN AL...	32	0.17	1	375	383
gb AAB86965.1 (AF032910) heat shock protein 58 [Leptos...	32	0.17	1	370	378
sp Q43831 RUBB_SECCE RUBISCO SUBUNIT BINDING-PROTEIN BE...	32	0.17	1	324	332
gb AAD04238.1 (AF014829) 60 kDa heat shock protein [Ba...	32	0.17	1	334	342
gb AAB97670.1 (AF005236) GroEL [Sitophilus oryzae prin...	32	0.17	1	371	379
sp P48212 CH60_CLOTM_60_KDA_CHAPERONIN (PROTEIN CPN60) ...	32	0.17	1	369	377
gb AAF91444.1 AF281650_1 (AF281650) heat shock protein ...	32	0.17	1	369	377
sp Q05972 CH61_SYNY3_60_KDA_CHAPERONIN_1 (PROTEIN CPN60)...	32	0.17	1	369	377
sp P09239 CH62_MYCLE_60_KDA_CHAPERONIN_2 (PROTEIN CPN60)...	32	0.17	1	369	377
pir B44425 chaperonin groEL - Synechocystis sp. (strai...	32	0.17	1	369	377
pir H72367 groEL protein - Thermotoga maritima (strain...	32	0.17	1	369	377
sp P42384 CH60_MYCPA_60_KDA_CHAPERONIN (PROTEIN CPN60) ...	32	0.17	1	369	377
sp P26209 CH60_BACP3_60_KD_CHAPERONIN (PROTEIN CPN60) (...)	32	0.17	1	369	377
sp P35471 CH63_RHIME_60_KDA_CHAPERONIN_C (PROTEIN CPN60)...	32	0.17	1	371	379
pir JN0511 heat shock protein groEL (clone Rhz B) - Rh...	32	0.17	1	371	379
emb CAA61520.1 (X89236) heat shock protein [Streptococ...	32	0.17	1	307	315
sp Q37757 CH60_CYPAP_60_KD_CHAPERONIN (PROTEIN CPN60) (...)	32	0.17	1	369	377
pir S61301 heat shock protein 63b - Neisseria gonorrhoe...	32	0.17	1	371	379
sp Q60024 CH60_THEBR_60_KDA_CHAPERONIN (PROTEIN CPN60) ...	32	0.17	1	369	377
emb CAB93056.1 (AL357432) chaperonin 2 [Streptomyces c...	32	0.17	1	369	377
gb AAD04244.1 (AF014835) 60 kDa heat shock protein [Ba...	32	0.17	1	277	285
sp P37578 CH61_MYCLE_60_KD_CHAPERONIN_1 (PROTEIN CPN60) ...	32	0.17	1	369	377
gb AAB65637.1 (U96734) GroEL [Bartonella henselae]	32	0.17	1	371	379
sp P43733 CH60_HAEIN_60_KD_CHAPERONIN (PROTEIN CPN60) (...)	32	0.17	1	371	379

sp P06139 CH60_ECOLI_60_KD_CHAPERONIN (PROTEIN CPN60) (...)	32	0.17	1	371	379
gb AAA25299.1 (M31918) htpB [Legionella pneumophila]	32	0.17	1	370	378
gb AAF7528.1 AF221845_1 (AF221845) GroEL [Vibrio parah...]	32	0.17	1	371	379
sp P95800 CH60_XANMA_60_KD_CHAPERONIN (PROTEIN CPN60) (...)	32	0.17	1	371	379
sp P48220 CH60_ZYMO_60_KD_CHAPERONIN (PROTEIN CPN60) (...)	32	0.17	1	370	378
pdb 1GRL Mol id: 1; Molecule: GroEL (Hsp60 Class); ...	32	0.17	1	371	379
pir S56371 GroEL protein - Escherichia coli >gi 742902...	32	0.17	1	371	379
sp P48217 CH60_SALTI_60_KD_CHAPERONIN (PROTEIN CPN60) (...)	32	0.17	1	371	379
dbj BAA92724.1 (AP001389) ESTs C99033(E4350), C99032(E4...	32	0.17	1	426	434
gb AAD04245.1 (AF014836) 60 kDa heat shock protein [Ba...	32	0.17	1	288	296
sp P46398 CH60_ACTAC_60_KDA_CHAPERONIN (PROTEIN CPN60) ...	32	0.17	1	371	379
sp O66200 CH60_ENTAG_60_KDA_CHAPERONIN (PROTEIN CPN60) ...	32	0.17	1	371	379
sp P26004 CH60_AMOPS_60_KDA_CHAPERONIN (PROTEIN CPN60) ...	32	0.17	1	370	378
sp O33964 CH60_BARQU_60_KDA_CHAPERONIN (PROTEIN CPN60) ...	32	0.17	1	371	379
sp P48218 CH60_STAEP_60_KDA_CHAPERONIN (PROTEIN CPN60) ...	32	0.17	1	369	377
gb AAB49990.1 (U90204) heat shock protein 60 [Tsukamur...	32	0.17	1	369	377
sp P26878 CH60_LEGPN_60_KDA_CHAPERONIN (PROTEIN CPN60) ...	32	0.17	1	369	377
sp O66202 CH60_SERRU_60_KDA_CHAPERONIN (PROTEIN CPN60) ...	32	0.17	1	371	379
pir B49855 heat shock protein GroEL - Bacillus stearot...	32	0.17	1	369	377
sp O66196 CH60_ENTAM_60_KDA_CHAPERONIN (PROTEIN CPN60) ...	32	0.17	1	371	379
sp Q07201 CH60_BACST_60_KDA_CHAPERONIN (PROTEIN CPN60) ...	32	0.17	1	369	377
pdb 1DER A Chain A, The 2.4 Angstrom Crystal Structure ...	32	0.17	1	370	378
emb CAB50775.1 (AJ243594) GroEL protein [Pseudoalterom...	32	0.17	1	372	380
pdb 1AON H Chain H, Crystal Structure Of The Asymmetric...	32	0.17	1	370	378
gb AAA53369.1 (U17072) GroEL [Pseudomonas aeruginosa]	32	0.17	1	371	379
sp P20110 CH61_RHOSH_60_KD_CHAPERONIN 1 (PROTEIN CPN60) ...	32	0.17	1	371	379
sp P48210 CH60_BORPE_60_KDA_CHAPERONIN (PROTEIN CPN60) ...	32	0.17	1	371	379
pir B39025 chaperonin 60 - Synechocystis sp. (PCC 6803...	32	0.17	1	370	378
sp P94166 CH60_ACTPL_60_KDA_CHAPERONIN (PROTEIN CPN60) ...	32	0.17	1	371	379
gb AAF83425.1 AE003907_3 (AE003907) 60kDa chaperonin [X...	32	0.17	1	371	379
pir B43606 heat shock protein groEL - Pseudomonas aeru...	32	0.17	1	371	379
sp P30718 CH60_PSEAE_60_KD_CHAPERONIN (PROTEIN CPN60) (...)	32	0.17	1	371	379

sp P31294 CH60_HAEDU 60 KD CHAPERONIN (PROTEIN CPN60) (...)	32	0.17	1	371	379
sp Q59573 CH61_MYCTU 60 KDA CHAPERONIN 1 (PROTEIN CPN60)...	32	0.17	1	369	377
sp Q66190 CH60_ENTAS 60 KDA CHAPERONIN (PROTEIN CPN60) ...	32	0.17	1	371	379
sp Q66192 CH60_ENTIT 60 KDA CHAPERONIN (PROTEIN CPN60) ...	32	0.17	1	371	379
pir B47292 heat shock protein groEL - Mycobacterium tu...	32	0.17	1	369	377
sp Q08854 CH60_STAAU 60 KDA CHAPERONIN (PROTEIN CPN60) ...	32	0.17	1	370	378
sp Q59687 CH60_PASMU 60 KDA CHAPERONIN (PROTEIN CPN60) ...	32	0.17	1	371	379
sp Q33963 CH60_BARHE 60 KDA CHAPERONIN (PROTEIN CPN60) ...	32	0.17	1	371	379
pdb 1OEL A Chain A, Mol_id: 1; Molecule: GroEL (Hsp60 C...	32	0.17	1	370	378
gb AAC24233.1 (AF071194) 60 kDa heat shock protein [Ba...	32	0.17	1	289	297
sp P35470 CH62_RHIME 60 KDA CHAPERONIN B (PROTEIN CPN60)...	32	0.17	1	371	379
gb AAF64160.1 AF239163_2 (AF239163) GroEL (Rhizobium le...	32	0.17	1	371	379
sp P19421 CH60_COXBU 60 KDA CHAPERONIN (PROTEIN CPN60) ...	32	0.17	1	371	379
sp P22034 CH62_SYNY3 60 KDA CHAPERONIN 2 (PROTEIN CPN60)...	32	0.17	1	371	379
sp Q33500 CH60_PSEST 60 KD CHAPERONIN (PROTEIN CPN60) (...)	32	0.17	1	371	379
sp P28256 CH60_CYACA 60 KD CHAPERONIN (PROTEIN CPN60) (...)	30	0.99	1	370I...	378
emb CAA65238.1 (X95992) heat shock protein 60 [Euglena...	29	1.4	1	386	..S.....	394
sp Q37683 CH60_TRYBB CHAPERONIN HSP60, MITOCHONDRIAL PR...	29	1.4	1	381	..S.....	389
sp Q39727 CH60_EUGGR CHAPERONIN CPN60, MITOCHONDRIAL PR...	29	1.4	1	385	..S.....	393
gb AAC68501.1 (AF030515) chaperonin 60 alpha subunit [...]	29	1.4	1	412	..S.....	420
sp P08824 RUBA_RICCO RUBISCO SUBUNIT BINDING-PROTEIN AL...	29	1.4	1	358	..S.....	366
pir S61397 heat shock protein hspB - Helicobacter pylor...	29	1.4	1	369	..S.....	377
sp Q69289 CH60_CAMJE 60 KDA CHAPERONIN (PROTEIN CPN60) ...	29	1.4	1	370	..S.....	378
emb CAA73778.1 (Y13334) heat shock protein [Campylobac...	29	1.4	1	370	..S.....	378
pir S19023 chaperonin groEL - Chlamydia pneumoniae >gi...	29	1.4	1	371	..S.....	379
sp P17203 CH60_CHLTR 60 KDA CHAPERONIN (PROTEIN CPN60) ...	29	1.4	1	371	..S.....	379
gb AAA97911.1 (U52049) GroEL [Chlamydia muridarum] >gi...	29	1.4	1	371	..S.....	379
sp P31681 CH60_CHLPN 60 KDA CHAPERONIN (PROTEIN CPN60) ...	29	1.4	1	371	..S.....	379
sp P56837 CH60_CHLMU 60 KD CHAPERONIN (PROTEIN CPN60) (...)	29	1.4	1	371	..S.....	379
sp P94798 CH60_FRATU 60 KDA CHAPERONIN (PROTEIN CPN60) ...	29	1.4	1	371	..S.....	379
gb AAB66326.1 (AF010281) GroEL [Lactobacillus zeae]	29	1.4	1	369V	377
sp P23033 CH60_TREPA 60 KDA CHAPERONIN (PROTEIN CPN60) ...	29	1.4	1	370	..S.....	378

sp P15599 CH60_CHLPS 60 KDA CHAPERONIN (PROTEIN CPN60) ...	29	1.4	1	371	..S.....	379
pir B41479 60K heat shock protein groEL - Chlamydia tr...	29	1.4	1	371	..S.....	379
pir A60273 60K heat shock protein groEL - Chlamydia tr...	29	1.4	1	371	..S.....	379
pir S33213 Tp4 antigen - Treponema pallidum >gi 297123...	29	1.4	1	370	..S.....	378
gb AAD34149.1 AF152236_2 (AF152236) chaperonin GroEL [M...	29	1.4	1	371	..S.....	379
gb AA773984.1 AF080547_2 (AF080547) GroEL protein [Clos...	29	1.4	1	369V	377
gb AA780372.1 AF159449_1 (AF159449) heat shock protein ...	29	1.4	1	369V	377
gb AAD23455.1 (AF117741) chaperonin GroEL [Streptococc...	29	1.4	1	369	..S.....	377
sp P30717 CH60_CLOAB 60 KDA CHAPERONIN (PROTEIN CPN60) ...	29	1.4	1	369V	377
sp P08823 RUBA_WHEAT RUBISCO SUBUNIT BINDING-PROTEIN AL...	29	1.4	1	371	..S.....	379
sp P34794 RUB2_BRANA RUBISCO SUBUNIT BINDING-PROTEIN AL...	29	1.4	1	413	..S.....	421
emb CAB51619.1 (X07852) rubisco subunit binding-protei...	29	1.4	1	358	..S.....	366
sp P21241 RUBB_BRANA RUBISCO SUBUNIT BINDING-PROTEIN BE...	29	1.4	1	425	..S.....	433
emb CAB91199.1 (AL355925) probable heat-shock protein ...	29	1.4	1	395	..S.....	403
pir S56645 chaperonin 60 alpha chain precursor, chloro...	29	1.4	1	404	..S.....	412
sp O60008 HS60_PARRR HEAT SHOCK PROTEIN 60 PRECURSOR (6...	29	1.4	1	409	..S.....	417
sp Q42694 RUBA_CHLRE RUBISCO SUBUNIT BINDING-PROTEIN AL...	29	1.4	1	404	..S.....	412
sp P94820 CH60_HOLOB 60 KDA CHAPERONIN (PROTEIN CPN60) ...	29	1.4	1	372	..S.....	380
gb AAD26368.1 AF130421_1 (AF130421) chaperonin GroEL [p...	29	1.4	1	371	..S.....	379
pir B71986 60Kda chaperone - Helicobacter pylori (stra...	29	1.4	1	370	..S.....	378
sp P42383 CH60_HELPY 60 KDA CHAPERONIN (PROTEIN CPN60) ...	29	1.4	1	370	..S.....	378
emb CAA52062.1 (X73840) heat shock protein 60 [Helicob...	29	1.4	1	370	..S.....	378
sp O74261 HS60_CANAL HEAT SHOCK PROTEIN 60 PRECURSOR (6...	29	1.4	1	388	..S.....	396
gb AAD16417.1 (AF100775) chaperonin 60.2 precursor [Le...	29	1.4	1	381	..S.....	389
sp P51349 CH60_FORPU 60 KD CHAPERONIN (PROTEIN CPN60) (...)	29	1.4	1	370	..S.....	378
gb AAC37260.3 (L43797) heat shock protein 60 [Trypanos...	29	1.4	1	381	..S.....	389
gb AAD16418.1 (AF100776) chaperonin 60.1 precursor [Le...	29	1.4	1	386	..S.....	394
gb AAF96718.1 (AB004410) chaperonin, 60 kd subunit [Vi...	29	1.4	1	371	..S.....	379
gb AAD00521.1 (U81786) heat-shock protein [Coccidioid...	29	1.4	1	413	..S.....	421
sp Q95046 CH60_TRYCR CHAPERONIN HSP60, MITOCHONDRIAL PR...	29	1.4	1	381	..S.....	389
pir S61295 heat shock protein 60 - Trypanosoma cruzi >...	29	1.4	1	381	..S.....	389
pir JT0901 chaperonin 60 beta precursor - Arabidopsis ...	29	1.4	1	425	..S.....	433

ref NP_013360.1	mitochondrial chaperonin, homolog of E...	29	1.4	1	393	..S.....	401
sp P21240 RUBB_ARATH	RUBISCO SUBUNIT BINDING-PROTEIN BE...	29	1.4	1	425	..S.....	433
sp P50142 HS60_AJECA	HEAT SHOCK PROTEIN 60 PRECURSOR (A...	29	1.4	1	409	..S.....	417
gb AAD10647.1	(AC005223) 52263 [Arabidopsis thaliana]	29	1.4	1	425	..S.....	433
sp P21238 RUBA_ARATH	RUBISCO SUBUNIT BINDING-PROTEIN AL...	29	1.4	1	415	..S.....	423
pir T07733	probable chaperonin 60 beta chain precursor...	29	1.4	1	424	..S.....	432
pir G75499	groEL protein - Deinococcus radiodurans (st...	29	1.4	1	369	..S.....	377
pir PS0374	chaperonin 60 beta (clone bx) - Arabidopsis...	29	1.4	1	2	..S.....	10
gb AAD26145.1 AF109791_1	(AF109791) 60 kD heat shock pr...	29	1.4	1	349	..S.....	357
sp P08926 RUBA_PEA	RUBISCO SUBUNIT BINDING-PROTEIN ALPH...	29	1.4	1	416	..S.....	424
gb AAD26143.1 AF109789_1	(AF109789) 60 kD heat shock pr...	29	1.4	1	349	..S.....	357
dbj BAB01754.1	(AP000603) GroEL protein; chaperonin, 6...	29	1.4	1	421	..S.....	429
gb AAB22560.2	(S40172) chaperonin homolog [Chlamydophi...	29	1.4	1	244	..S.....	252
sp P08927 RUBB_PEA	RUBISCO SUBUNIT BINDING-PROTEIN BETA...	29	1.4	1	419	..S.....	427
sp Q94596 CH60_LEIMA	CHAPERONIN HSP60, MITOCHONDRIAL PR...	29	1.4	1	386	..S.....	394
sp P26821 CH60_CLOPE	60 KDA CHAPERONIN (PROTEIN CPN60) ...	29	1.4	1	369V	377
sp P37282 CH60_LACLA	60 KDA CHAPERONIN (PROTEIN CPN60) ...	29	1.4	1	369V	377
gb AAB06712.1	(U52923) cpn-60 [Trichomonas vaginalis]	29	2.0	1	315	..T.....	323
gb AAB39487.1	(U57000) chaperonin 60 [Trichomonas vagi...	29	2.0	1	372	..T.....	380
gb AAC29004.1	(AF031929) chaperonin GroEL [Lactobacill...	29	2.0	1	369	..T.....	377
sp P95647 CH62_RHOSH	60 KD CHAPERONIN 2 (PROTEIN CPN60) ...	29	2.0	1	371	378
sp Q95058 CH60_TRIVA	HYDROGENOSOMAL CHAPERONIN HSP60 (P...	29	2.0	1	385	..T.....	393
sp P42375 CH60_PORGI	60 KDA CHAPERONIN (PROTEIN CPN60) ...	28	2.9	1	371	378
pir S47530	chaperonin groEL - Porphyromonas gingivalis...	28	2.9	1	371	378
emb CAA37994.1	(X54059) common antigen [Borrelia burgd...	28	2.9	1	369	..V.....	377
pir PW0005	chaperonine 60K alpha chain - rape (fragment)	28	2.9	1	375	..P.....	383
sp P27575 CH60_BORBU	60 KDA CHAPERONIN (PROTEIN CPN60) ...	28	2.9	1	369	..V.....	377
pir S16428	heat shock protein 60 - Lyme disease spiroc...	28	2.9	1	369	..V.....	377
gb AAB65633.1	(U96732) GroEL [Ehrlichia risticii]	28	2.9	1	372	379
pir JC6063	chaperonin groEL - Bacillus sp	28	2.9	1	369	376
pir S70013	chaperonin-like protein groEL2 - Synecococ...	28	2.9	1	369	..V.....	377
emb CAB43992.1	(AJ006516) heat shock protein 60 [Bacte...	28	2.9	1	371	378

sp O50305 CH60_BACHD 60 KDA CHAPERONIN (PROTEIN CPN60) ...	282.9	1	369	376
gb AAD37976.1 AF145252_2 (AF145252) heat shock protein ...	282.9	1	371	378
sp O66222 CH60_ERWAP 60 KDA CHAPERONIN (PROTEIN CPN60) ...	282.9	1	371	378
sp O66216 CH60_ERWHE 60 KDA CHAPERONIN (PROTEIN CPN60) ...	282.9	1	371	378
sp O66218 CH60_PANAN 60 KDA CHAPERONIN (PROTEIN CPN60) ...	282.9	1	371	378
sp O32606 CH60_EHRSE 60 KDA CHAPERONIN (PROTEIN CPN60) ...	282.9	1	372	379
sp P48214 CH60_EHRI 60 KDA CHAPERONIN (PROTEIN CPN60) ...	282.9	1	372	379
gb AAC36500.1 (U45241) GroEL/HSP60 homolog [Lawsonia i...	282.9	1	371	..V.....	379
pir S52901 heat shock protein 60K - Yersinia enterocol...	284.1	1	371X...	379
sp O66026 CH60_KLEPN 60 KDA CHAPERONIN (PROTEIN CPN60) ...	284.1	1	370	..V.....	378
sp O67943 CH60_AQUAE 60 KDA CHAPERONIN (PROTEIN CPN60) ...	278.3	1	371	..S....I.	379
dbj BAA09171.1 (D50609) heat-shock protein (HSP60) [Sc...	278.3	1	403	..S..I...	411
sp Q09864 HS60_SCHPO HEAT SHOCK PROTEIN 60 PRECURSOR (H...	278.3	1	403	..S..I...	411
sp P49464 CH60_ODOSI 60 KD CHAPERONIN (PROTEIN CPN60) (...	278.3	1	371	..S..I...	379
gb AAF12968.1 AF022186_91 (AF022186) unknown; 60 kd cha...	278.3	1	370	..I.....	378
gb AAD26144.1 AF109790_1 (AF109790) 60 kd heat shock pr...	278.3	1	349	..S..I...	357
pir H72761 hypothetical protein APE0085 - Aeropyrum pe...	26	12	76	..L.....	84
sp P22879 CH60_SYNP7 60 KDA CHAPERONIN (PROTEIN CPN60) ...	26	12	369	..S....V	377
sp P40171 CH61_STRCO 60 KD CHAPERONIN 1 (PROTEIN CPN60 ...	26	12	370C...	378
sp Q00767 CH61_STRAL 60 KD CHAPERONIN 1 (PROTEIN CPN60 ...	26	12	371C...	379
emb CAA65225.1 (X95970) GroEL protein [Streptomyces l...	26	12	370C...	378
pir T35591 chaperonin cpn60 - Streptomyces coelicolor ...	26	12	371C...	379
gb AAD44540.2 AF115542_1 (AF115542) GroEL-type chaperon...	26	12	362I...	369
dbj BAA22519.1 (D10972) GroEL protein [Bacillus subtilis]	26	17	369R...	377
prf 1906220B groEL gene [Bacillus subtilis]	26	17	369R...	377
dbj BAA88110.1 (AB028452) Cpn60 [Bacillus sp. MS]	26	17	369R...	377
sp Q05046 CH62_CUCMA CHAPERONIN CPN60-2, MITOCHONDRIAL ...	25	24	402	..S.....	409
pir H69367 hypothetical protein AF0944 - Archaeoglobus...	25	24	359	365
dbj BAA94828.1 (AB032711) heat shock protein [Ehrlich...	25	24	373	..S.....	380
sp O34337 CH60_EHRPH 60 KDA CHAPERONIN (PROTEIN CPN60) ...	25	24	372	..S.....	379
dbj BAA94829.1 (AB032712) heat shock protein [Ehrlich...	25	24	373	..S.....	380
sp Q05045 CH61_CUCMA CHAPERONIN CPN60-1, MITOCHONDRIAL ...	25	24	402	..S.....	409

sp O85754 CH60_RICTY 60 KDA CHAPERONIN (PROTEIN CPN60) ...	25	24	371	..S.....	378
sp P29185 CH61_MAIZE CHAPERONIN CPN60-1, MITOCHONDRIAL ...	25	24	404	..S.....	411
pir S26582 chaperonin hsp60 - maize >gi 22248 emb CAA7... ..	25	24	404	..S.....	411
dbj BAB03017.1 (AP001297) chaperonin hsp60, mitochondr...	25	24	401	..S.....	408
pir S20875 chaperonin hsp60 precursor - maize >gi 2224... ..	25	24	404	..S.....	411
sp P29197 CH60_ARATH CHAPERONIN CPN60, MITOCHONDRIAL PR...	25	24	401	..S.....	408
gb AAA33450.1 (U21007) chaperonin 60 [Zea mays]	25	24	404	..S.....	411
gb AAF03899.1 AF195273_1 (AF195273) chaperonin GroEL [E...	25	24	373	..S.....	380
gb AAD45382.1 AF165812_1 (AF165812) GroEL [Anaplasma ma...	25	24	373	..S.....	380
gb AAF86372.1 AF210459_1 (AF210459) chaperonin GroEL [E...	25	24	373	..S.....	380
sp Q00768 CH62_STRAL 60 KD CHAPERONIN 2 (PROTEIN CPN60)...	25	24	371	377
sp O34194 CH60_EHRCA 60 KDA CHAPERONIN (PROTEIN CPN60) ...	25	24	373	..S.....	380
sp P42382 CH60_EHRCH 60 KDA CHAPERONIN (PROTEIN CPN60) ...	25	24	373	..S.....	380
emb CAB40143.1 (Y15783) chaperonin hsp60, GroEL [Ricke...	25	24	371	..S.....	378
sp O34191 CH60_EHREQ 60 KDA CHAPERONIN (PROTEIN CPN60) ...	25	24	372	..S.....	379
sp Q9ZCT7 CH60_RICPR 60 KDA CHAPERONIN (PROTEIN CPN60) ...	25	24	371	..S.....	378
emb CAA70570.1 (Y09416) heat shock protein 60 [Onchoce...	25	24	374	..S.....	381
gb AAC04902.1 (AC002334) mitochondrial chaperonin (HSP...	25	24	341	..S.....	348
gb AAC38819.1 (AF029366) chaperonin 60 [Entamoeba hist...	25	24	377	T.....	384
gb AAD51014.1 (AF172158) heat shock protein GroEL [Ehr...	25	24	372	..S.....	379
gb AAD51016.1 (AF172159) heat shock protein GroEL [Ehr...	25	24	372	..S.....	379
pir T22097 hypothetical protein W07G4.6 - Caenorhabdit...	25	24	62	T.....	69
gb AAC41576.1 (L39934) cpn60 gene product [Entamoeba h...	25	24	322	T.....	329
sp O34198 CH60_RICRI 60 KDA CHAPERONIN (PROTEIN CPN60) ...	25	24	371	..S.....	378
gb AAB47483.1 (U77379) GroEL-related molecular chapero...	25	24	371	..S.....	378
sp P25750 CH60_BUCAI 60 KDA CHAPERONIN (PROTEIN CPN60) ...	25	24	371	..S.....	378
sp P35480 CH60_BRANA CHAPERONIN CPN60, MITOCHONDRIAL PR...	25	24	405	..S.....	412
gb AAB47482.1 (U77380) GroEL-related molecular chapero...	25	24	372	..S.....	379
dbj BAB02911.1 (AB028610) chaperonin; similar to GroEL...	25	24	401	..S.....	408
sp O51832 CH60_BUCMP 60 KDA CHAPERONIN (PROTEIN CPN60) ...	25	24	371	..S.....	378
emb CAA65226.1 (X95971) GroEL2 protein [Streptomyces l...	25	24	371	377
sp Q43298 CH62_MAIZE CHAPERONIN CPN60-2, MITOCHONDRIAL ...	25	24	404	..S.....	411

pir S26583	chaperonin hsp60 - maize >gi 22250 emb CAA7...	25	2	4	404	..S.....	411
gb AA07941.1 AF192796.1	(AF192796) GroEL [Ehrlichia ph...]	25	2	4	372	..S.....	379
gb AAC17990.1	(AF033101) heat shock protein [Ehrlichia...]	25	2	4	372	..S.....	379
sp Q59177 CH60_BUCAP_60	KDA CHAPERONIN (PROTEIN CPN60) ...	25	2	4	374	..S.....	381
sp P48213 CH60_COWRU_60	KDA CHAPERONIN (PROTEIN CPN60) ...	25	2	4	373	..S.....	380
dbj BAAL9540.1	(AB002286) similar to GroEL protein [Wo...]	25	2	4	376	..S.....	383
pir S78480	Rhesus blood group antigen-like protein iso...	25	3	4	275	281
sp Q28446 RHL_HYLP1	RH-LIKE PROTEIN (RHESUS-LIKE PROTEI...)	25	3	4	275	281
pir H70505	hypothetical protein Rv2729c - Mycobacteriu...	25	3	4	113	..A....	120
gb AAF37156.1 AF132980.1	(AF132980) Rh50 [Cebus apella]	25	3	4	274	280
gb AAG00304.1	(AF177622) Rh50 glycoprotein [Gorilla go...]	25	3	4	277	283
gb AAF23099.1	(AF179684) mutant Rh50 glycoprotein [Hom...]	25	3	4	277	283
gb AAC04247.1	(AF031548) erythrocyte membrane glycopro...	25	3	4	277	283
gb AAG00303.1	(AF177621) Rh50 glycoprotein [Pan troglo...]	25	3	4	277	283
ref NP_000315.1	Rhesus blood group-associated glycopro...	25	3	4	277	283
gb AAF23100.1	(AF179685) mutant Rh50 glycoprotein [Hom...]	25	3	4	277	283
gb AAC18092.1	(AF056965) mutant membrane protein Rhce ...	25	3	4	275	281
gb AAC94964.1	(AF012427) RH30-like protein [saimiri bo...]	25	3	4	217	223
emb CAA73029.1	(Y12397) Rh antigen-like protein [Geodi...]	25	3	4	290	296
emb CAB10169.1	(Z97333) RHCE protein [Homo sapiens]	25	3	4	63	69
gb AAD13385.1	(AF058917) Rh50-like protein [Macaca mul...]	25	3	4	277	283
gb AAD56366.1	(AF178842) Rhesus blood group-associated...	25	3	4	8	14
dbj BAA32444.1	(AB015467) 50 kD glycoprotein (Rh50) [M...]	25	3	4	277	283
emb CAB10170.1	(Z97334) RHD protein [Homo sapiens]	25	3	4	63	69
gb AAG00308.1	(AF177626) Rh50 glycoprotein [Papio hama...]	25	3	4	277	283
gb AAC94967.1	(AF012430) RH30-like protein [Mus musculus]	25	3	4	265	271
gb AAF22501.1 AF101479_1	(AF101479) Rh30-like protein [...]	25	3	4	275	281
gb AAC94968.1	(AF021845) RH30-like polypeptide [Eulemu...]	25	3	4	89	95
gb AAG00306.1	(AF177624) Rh50 glycoprotein [Hylobates ...]	25	3	4	277	283
gb AAC94966.1	(AF012429) RH30-like protein [Callithrix...]	25	3	4	275	281
gb AAF22442.1 AF052588_1	(AF052588) Rh-like protein [Ca...]	25	3	4	275	281
gb AAC94965.1	(AF012428) RH30-like protein [saimiri sc...]	25	3	4	275	281

pir S78478	Rhesus blood group antigen-like protein iso...	25	34	170	176
gb AAG00305.1	(AF177623) Rh50 glycoprotein [Pongo pygm...	25	34	277	283
gb AAG00307.1	(AF177625) Rh50 glycoprotein [Macaca mul...	25	34	277	283
ref NP_057405.1	Rh type C glycoprotein >gi 5911316 gb ...	25	34	287	293
gb AAB34659.1	RhPI-Id-Rhesus blood group antigen isofo...	25	34	135	141
pir I52589	erythrocyte membrane protein Rh (Rhesus) D ...	25	34	275	281
dbj BAA32440.1	(AB015191) Rh blood group protein [Ratt...	25	34	282	288
gb AAC01711.1	(AF040570) polyketide synthase [Amycolat...	25	34	191	2	1918
gb AAC94962.1	(AF012425) RH30-like protein [Pongo pygm...	25	34	275	281
gb AAC94963.1	(AF012426) RH30-like protein [Papio hama...	25	34	275	281
pir I52596	RhK562-II - human >gi 693867 gb AAB31911.1 ...	25	34	275	281
dbj BAA32438.1	(AB015189) Rh blood group protein [Mus ...	25	34	278	284
ref NP_035399.1	Rhesus blood group-associated A glycop...	25	34	287	293
ref NP_035400.1	Rhesus blood group-like >gi 3319280 gb ...	25	34	278	284
gb AAD55410.1	AF181624_1 (AF181624) BcDNA.GH03016 [Dros...	25	34	281	287
dbj BAA32442.1	(AB015193) 50 kD glycoprotein (Rh50) [M...	25	34	287	293
dbj BAA32439.1	(AB015190) Rh blood group protein [Mus ...	25	34	278	284
gb AAB26081.1	(S57971) Rh polypeptide II [clone RHP11]...	25	34	275	281
gb AAB66508.1	(U78289) ty lactone synthase module 7 [St...	25	34	233	239
sp Q28426 RHLC_GORGO	RH-LIKE PROTEIN IC (RHESUS-LIKE PR...	25	34	275	281
dbj BAA33927.1	(AB018644) Rh blood CE group antigen po...	25	34	275	281
dbj BAA90298.1	(AB037270) Rh blood group antigen Rhd [...	25	34	275	281
gb AAC04248.1	(AF031549) mutant erythrocyte membrane g...	25	34	277	283
sp Q28813 RHLA_PANTR	RH-LIKE PROTEIN IA (RHESUS-LIKE PR...	25	34	275	281
sp Q28427 RHLD_GORGO	RHESUS-LIKE PROTEIN >gi 2134608 pi...	25	34	275	281
pir S40517	erythrocyte membrane protein - human	25	34	275	281
pir I52615	gene Rhd protein - human >gi 999310 gb AAB3...	25	34	275	281
dbj BAA81899.1	(AB018966) Rh blood group D antigen (Rh...	25	34	275	281
dbj BAA82627.1	(AB030388) Rh blood CE group antigen po...	25	34	275	281
sp Q02161 RHD_HUMAN	BLOOD GROUP RH(D) POLYPEPTIDE (RHES...	25	34	275	281
sp F18577 RHCE_HUMAN	BLOOD GROUP RH(CE) POLYPEPTIDE (RH...	25	34	275	281
emb CAB75731.1	(AJ287289) Rhesus blood group, D antige...	25	34	275	281

pir A46368 erythrocyte membrane protein Rh (Rhesus) D ...	25	34	275	281
gb AAD11526.1 (U59270) rhesus-like protein [Bos taurus]	25	34	275	281
sp Q28812 RHLF_PANTR RH-LIKE PROTEIN IIF (RHESUS-LIKE P...	25	34	275	281
sp Q28814 RHLR_PANTR RH-LIKE PROTEIN IIR (RHESUS-LIKE P...	25	34	275	281
ref NP_057208.1 Rh blood group D antigen (RHD) >gi 536...	25	34	275	281
ref NP_057309.1 RHD type IIIa protein >gi 6164859 gb A...	25	34	275	281
gb AAB37696.2 (S82449) D category IIIC antigen [Homo s...	25	34	275	281
dbj BAAB2159.1 (AB018969) Rh blood group D antigen (RH...	25	34	275	281
dbj BAA33928.1 (AB018645) Rh blood group CE antigen po...	25	34	275	281
dbj BAAB1900.1 (AB018967) Rh blood group D antigen (RH...	25	34	275	281
prf 2021440A Rh protein [Homo sapiens]	25	34	275	281
pir S40515 erythrocyte membrane protein - human	25	34	275	281
pir S40516 erythrocyte membrane protein - human	25	34	275	281
sp Q28481 RHL_MACFA RH-LIKE PROTEIN (RHESUS-LIKE PROTEI...	25	34	275	281
ref NP_057275.1 Rh blood group antigen Evans >gi 59113...	25	34	275	281
emb CAB09722.1 (Z97026) rhesus D category VI type III ...	25	34	275	281
gb AAC46028.1 (AF016585) polyketide synthase module 7 ...	25	34	235	241
emb CAB72300.1 (AL031284) dJ469D22.1 (Rhesus blood gro...	25	34	296	302
pir S73004 hypothetical protein B229_F2_64 - Mycobacte...	25	34	38	44
gb AAF04566.1 (AF187847) truncated Rh50 glycoprotein [...	25	34	270	276
pir T17409 polyketide synthase type I - Streptomyces v...	25	34	130	4 1310	
gb AAD23959.1 AF102876_1 (AF102876) Rh blood group poly...	25	34	279	9 2804	
pir T17464 rifamycin polyketide synthase modules 4-6 -...	25	34	268 274	
sp Q59228 AAT_BACST ASPARTATE AMINOTRANSFERASE (TRANSAM...	24	49	192	1 1927	
gb AAB17277.1 (U72247) chaperonin 60 [Dictyostelium di...	24	49	132P. 139	
sp P23034 AAT_BACSP ASPARTATE AMINOTRANSFERASE (TRANSAM...	24	49	387	.IG..... 395	
pir A75054 molybdenum cofactor biosynthesis protein (m...	24	49	134P. 141	
pir S56644 chaperonin 60 beta-1 chain - Chlamydomonas ...	23	100	353	.E..... 360	
sp Q42693 RUBB_CHLRE RUBISCO SUBUNIT BINDING-PROTEIN BE...	23	100	256	.S.....I. 263	
sp O31151 UVRA_ZYMO EXCINUCLEASE ABC SUBUNIT A >gi 746...	23	100	256	.S.....I. 263	
pir T26132 hypothetical protein W04A4.4 - Caenorhabdit...	23	100	227I.I. 234	
sp Q42695 RUBC_CHLRE RUBISCO SUBUNIT BINDING-PROTEIN BE...	23	100	61	.S.....G. 69	

gb AAB06711.1 (U52924) cpn-60 [Naegleria fowleri]	23	143	87	S....I.	94
sp P23214 OAC_BPSF6 O-ACETYL TRANSFERASE (O-ANTIGEN ACE...	23	143	316	.S....I	323
pir JS0588 O-antigen acetylase (EC 2.3.1.-) - enteroba...	23	143	40	...I...	46
pir T28882 protein SUP-10 precursor - Caenorhabditis e...	23	143	40	...I...	46
pir B75611 urocanate hydratase - Deinococcus radiodura...	23	143	307	T..I...	314
gb AAC77915.1 (AF063588) enoyl CoA hydratase [Rhodococ...	23	143	184SI	191
gb AAF83179.1 AE003888_12 (AE003888) fimbrial assembly ...	22	203	31	...I...	37
gb AAF97865.1 (AF183391) Rh-like protein-2 [Caenorhabd...	22	203	331C...	338
pir T29442 hypothetical protein F08F3.3 - Caenorhabdit...	22	203	279I	285
pir C69540 cobalamin (5'-phosphate) synthase (cobS-2) ...	22	203	279I	285
pir D70000 conserved hypothetical protein ytrF - Bacill...	22	203	99V	105
emb CAA05643.1 (AJ002638) methyltransferase [Streptomy...	22	203	409	414
gb AAA65602.1 (U24396) heat-shock protein [Ehrlichia r...	22	203	141	146
gb AAA84990.1 (U41754) NfeD [Rhizobium etli] >gi 26553...	22	203	334G...	341
pir T18673 hypothetical protein B0240.1 - Caenorhabdit...	22	203	351	356
gb AAF32412.1 (AF069392) flagellar biosynthesis protei...	22	203	275I	281
gb AAF84712.1 AE004010_9 (AE004010) conserved hypotheti...	22	203	33	V.....IV	41
gb AAF47296.1 (AE003465) anon-EST.fe2C9 gene product [...	22	203	90	95
gb AAB81488.1 (AF005857) anon2C9 [Drosophila melanogas...	22	203	50V	56
dbj BAA99699.1 (AB025624) contains similarity to Na+/H...	22	203	50V	56
sp P49609 ACON_GRAVE ACONITATE HYDRATASE, MITOCHONDRIAL...	22	203	277	..L...V..	285
emb CAA76360.1 (Y16747) aconitate hydratase [Piromyces...	22	203	680	685
gb AAF19813.1 AF188287_5 (AF188287) MtaE [Stigmatella a...	22	203	655	660
gb AAB81489.1 (AF005858) anon2C9 [Drosophila yakuba]	22	203	236S...	242
gb AAC12772.1 (AF056082) chaperonin 60; cpn60 [Leishma...	22	203	53V	59
gb AAC13642.1 (AF029733) unknown [Xanthobacter autotro...	22	203	328	333
gb AAD38419.1 (AF065609) heat shock protein 60 [Toxopl...	22	203	24	29
emb CAB76251.1 (X82447) CoxK protein [Oligotropha carb...	22	203	398	403
emb CAA73085.1 (Y12482) glutamine--pyruvate aminotrans...	22	203	282	287
sp Q92519 PGK_STRCO PHOSPHOGLYCERATE KINASE >gi 7480321...	22	203	1402	1407
gb AAF95516.1 (AE004307) glutamate synthase, large sub...	22	203	110	115
sp P27100 TCBE_PSESQ CARBOXYMETHYLENEBUTENOLIDASE (DIEN...	22	203	1418	1423

dbj BAA74533.1	(AB019032) diene lactone hydrolase [Rals...	22	203	115	120
gb AA85127.1	(U42203) third gene in flhQ operon; memb...	22	290	115	120
dbj BAA84474.1	(AB032367) type I polyketide synthase A...	22	290	40	...A.S...	47
gb AAF89838.1	AF173961_4 (AF173961) BbsC [Thauera aroma...	22	290	680S.	686
pdb 1KID	Groel (Hsp60 Class) Fragment (Apical Domai...	22	290	2281T.	2287
pir E70885	hypothetical protein RV2859c - Mycobacteriu...	22	290	199IP.	206
gb AAF6396.1	AF235504_17 (AF235504) Fkba [Streptomyces...	22	290	198	203
sp P35825 SLAP_BACST	S-LAYER PROTEIN PRECURSOR (SURFACE...	22	290	99	M.....	105
gb AAF51629.1	(AE003592) kni gene product [Drosophila ...	22	290	3633T.	3639
sp Q08870 PLPC_PASHA	OUTER MEMBRANE LIPOPROTEIN 3 PRECU...	22	290	5566S.	5572
pir T30283	polyketide synthase - Streptomyces sp. (str...	22	290	513	518
gb AAF05308.1	AF171228_1 (AF171228) pectate lyase [Pect...	22	290	81S.	87
pir S69796	pectate lyase (EC 4.2.2.2) precursor - Erwi...	22	290	5	...A..I	12
sp Q47473 PELL_ERWCH	PECTATE LYASE L PRECURSOR (PECTATE...	22	290	3674T.	3680
dbj BAB03374.1	(AP002486) hypothetical protein [oryza ...	22	290	5601S.	5607
sp P10734 KNIR_DROME	ZYGOTIC GAP PROTEIN KNIRPS >gi 815...	22	290	304T..	310
gb AAF49901.1	(AE003541) CG10627 gene product [Drosoph...	22	290	304T..	310
dbj BAB01222.1	(AB016889) alcohol dehydrogenase-like p...	22	290	304T..	310
sp P54957 YXER_BACSU	HYPOTHETICAL 38.4 KD PROTEIN IN ID...	22	290	228S.	233
gb AAF19812.1	AF188287_4 (AF188287) MtaD [Stigmatella a...	22	290	107S.	113
sp Q58936 YF41_METJA	HYPOTHETICAL PROTEIN MJ1541 >gi 21...	22	290	531	536
gb AAC38669.2	(AF062345) putative acetyltransferase; L...	22	290	35	..E.K...	43
pir D72655	hypothetical protein APE0671 - Aeropyrum pe...	22	290	339	344
gb AAA25548.1	(L16627) lipoprotein 3 [Pasteurella haem...	22	290	1651S.	1657
gb AAC05488.1	(AF047326) chromomethylase [Gaultheria s...	22	290	269	...S...P.	277
gb AAC05487.1	(AF047325) chromomethylase [Rhododendron...	22	290	157S.	163
pir G70944	probable polyketide synthase RV2048c - Myco...	21	414	76M..V	83
gb AAB66506.1	(U78289) ty lactone synthase modules 4 & ...	21	414	2	...A..I	9
dbj BAA84475.1	(AB032367) type I polyketide synthase A...	21	414	46	51
pir T17410	polyketide synthase type I - Streptomyces v...	21	414	46	51
dbj BAA84479.1	(AB032367) type I polyketide synthase A...	21	414	226T.	232
dbj BAA94031.1	(AB034704) hypothetical soluble protein...	21	414	2249T.	2255

gb AAC45407.1	(U87980)	MadL [Malonomonas rubra]	21	41	4	237T.	243
gb AAF62883.1	AF217189_6	(AF217189) epoD [Sorangium cel...]	21	41	4	127	8T.	1284
gb AAF71766.1	AF263912_5	(AF263912) NysI [Streptomyces ...]	21	41	4	446	8T.	4474
sp Q03132 ERY2_SACER	ERYTHRONOLIDE SYNTHASE, MODULES 3 ...		21	41	4	231T.	237
gb AAF26921.1	AF210843_18	(AF210843) polyketide synthas...	21	41	4	173	5T.	1741
emb CAA44448.1	(X62569) 6-deoxyerythronolide B synthas...		21	41	4	229T.	235
gb AAF72076.1	AC025098_10	(AC025098) hypothetical prote...	21	41	4	178	6T.	1792
pir T17428	FK506 polyketide synthase - Streptomyces sp...		21	41	4	283	1T.	2837
pir T17467	rifamycin polyketide synthase modules 9-10 ..		21	41	4	12G..	18
sp P96190 TPIS_XANFL	TRIOSEPHOSPHATE ISOMERASE (TIM) >g...		21	41	4	96	101
sp P55203 CYGD_BOVIN	RETINAL GUANYLYL CYCLASE 1 PRECURS...		21	41	4	529	6T.	5301
gb AAD12319.1	(U77104) membrane guanylate cyclase isof...		21	41	4	200	6T.	2012
pir E70522	probable polyketide synthase - Mycobacteriu...		21	41	4	809	5T.	8101
emb CAA06231.1	(AJ004933) periplasmic nitrate reductas...		21	41	4	168	4T.	1690
gb AAA36751.1	(J03593) ADP.ATP translocase [Homo sapiens]		21	41	4	529	6T.	5301
gb AAB66504.1	(U78289) ty lactone synthase starter modu...		21	41	4	168	4T.	1690
pir S73074	pkS002b protein - Mycobacterium tuberculosi...		21	41	4	189	194
gb AAD03047.1	(AF098795) type-I polyketide synthase; C...		21	41	4	137	3T.	1379
sp P74178 YB78_SINY3	HYPOTHETICAL 69.1 KD PROTEIN SLL11...		21	41	4	189	5T.	1901
gb AAF15393.1	AF109176_1	(AF109176) NAD dependent gluta...	21	41	4	87	..A....	93
gb AAF13178.1	AF181079_1	(AF181079) sensor kinase homol...	21	41	4	362	367
pir S23070	erythronolide synthase (EC 2.3.1.94) II - S...		21	41	4	362	367
gb AAF23206.1	AC016795_19	(AC016795) putative amino aci...	21	41	4	219	224
emb CAB60885.2	(AL132880) predicted using Genefinder; ...		21	41	4	22	27
prf 1702361A	polyketide synthase [Saccharopolyspora ex...		21	41	4	13	18
pir A71479	probable rod shape protein - Chlamydia trac...		21	41	4	125	5T.	1261
ref NP_056495.1	hypothetical protein >gi 4929583 gb AA...		21	41	4	277	1T.	2777
pir T36494	probable membrane protein - Streptomyces co...		21	41	4	239T.	245
gb AAC26135.1	(AF074603) nonactate synthase [Streptomy...		21	41	4	801T.	807
pir T30225	polyketide synthase - Streptomyces hygrosco...		21	41	4	308	313
sp Q28849 RHL_MACMU	RH-LIKE PROTEIN (RHESUS-LIKE PROTEI...		21	41	4	365	370
emb CAA75437.1	(Y15166) NADP-glutamate dehydrogenase [...]		21	41	4	45	50

gb AAF95161.1	(AE004276) PTS system, glucose-specific ...	21	41	4	169	3T.	1699
gb AAB82307.1	(U39783) amino acid transport protein [A...	21	41	4	223		228
sp Q03133 ERY3_SACER	ERYTHRONOLIDE SYNTHASE, MODULES 5 ...	21	41	4	433P..		439
emb CAB61936.1	(AL133278) putative dehydrogenase/reduc...	21	41	4	222T.		228
gb AAF38979.1	(AE002277) cell shape-determining protei...	21	41	4	318T.		324
gb AAF63256.1	(AF209468) Rh50-like protein [Danio rerio]	21	41	4	151		156
emb CAC01546.1	(AL391039) putative delta fatty acid de...	21	41	4	200		205
pir H70559	hypothetical protein Rv1634 - Mycobacterium...	21	41	4	43		48
pir T00962	hypothetical protein F20D22.12 - Arabidopsi...	21	41	4	345	5.....T.		3461
emb CAB83925.1	(AL162753) hypothetical protein NMA0635...	21	41	4	275		280
gb AAD24191.1	AF133718_1 (AF133718) cell cycle histidin...	21	41	4	372		377
pir E75262	2-oxo acid dehydrogenase, lipoamide dehydro...	21	41	4	138		143
ref NP_055565.1	KIAA0008 gene product >gi 3183208 sp Q...	21	41	4	223		228
pir H75035	probable membrane protein PAB2428 - Pyrococ...	21	41	4	222T.		228
gb AAF42159.1	(AE002531) conserved hypothetical protei...	21	41	4	3	..D.K....		11
pir S52053	cytochrome-c oxidase (EC 1.9.3.1) chain I -...	21	41	4	318T.		324
gb AAC44061.1	(U51197) glycosyl transferase [sphingomo...	21	41	4	284T.		290
emb CAB82454.1	(AJ276513) Cnrt protein [Ralstonia.sp....	21	41	4	52		57
gb AAF50117.1	(AE003546) CG11806 gene product [Drosoph...	21	41	4	25		30
pir S13595	6-deoxyerythronolide B synthase - Saccharop...	21	41	4	430M.		436
pir S04904	glutamate dehydrogenase (NADP+) (EC 1.4.1.4...	21	41	4	181	...M....		187
gb AAF62099.1	AF243130_1 (AF243130) EM88 antigen [Mus m...	21	41	4	316		321
sp P18819 DHE4_EMENI	NADP-SPECIFIC GLUTAMATE DEHYDROGEN...	21	41	4	111	..T....		117
					648		653
					60		65
					181	...M....		187
					204TM.		211
					100		105
					82		87
					148		153
					222T.		228
					379		384

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22, 1315-22.

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WHAT IS CLAIMED IS:

1. An isolated peptide of at least 8 amino acid residues in length and having an amino acid sequence at least 75% identical to the
5 sequence selected from the group consisting of SEQ ID NOS: 2, 3, 4, 7, 17 and 31.
2. An isolated peptide of no more than about 11 amino acids in length comprising a sequence selected from the group consisting of SEQ ID NOS: 2, 3, 4, 7, 17 and 31.
- 10 3. An isolated nonapeptide having an amino acid sequence differing by no more than about 2 amino acids from the sequences selected from the group consisting of SEQ ID NOS: 2, 3, 4 and 31.
4. An isolated nonapeptide having a sequence differing by no more than 1 amino acid from a sequence selected from the group consisting of
15 SEQ ID NOS: 2, 3, 4 and 31.
5. The isolated nonapeptide of claim 4 wherein the amino acid difference is a substitution of one conservative amino acid for another.
6. An immunogen or immunogenic composition comprising one or more isolated peptides selected from the group consisting of the peptides
20 of claims 1, 2, 3, 4, and 5.
7. An immunogen or immunogenic composition comprising a polypeptide of the amino acid sequence of SEQ ID NO:4.
8. An immunogen or immunogenic composition comprising a polypeptide, other than mycobacterial heat shock protein 65 (Mhsp65) or
25 a protein homologous to human heat shock protein, wherein said

polypeptide comprises one or more epitopes selected from the peptides of claims 1 through 5, and wherein each said epitopic peptide may be present in one or more copies.

9. An immunogen or immunogenic composition comprising a polynucleotide encoding a CTL-specific epitope wherein said epitope is selected from a group consisting of the peptides of claims 1, 2, 3, 4, and 5 and operably linked to a promoter, and wherein the polynucleotide sequence does not include the entire Mhsp65 gene or a gene highly homologous to the human heat shock protein 65 gene.

10. The immunogen of claim 9 wherein said immunogen is a plasmid comprising said polynucleotide encoding a CTL-specific epitope.

11. A vaccine composition comprising an immunologically active amount of the immunogens of claims 6, 7, 8, 9 and 10.

12. A method for inducing a CTL response *in vitro* that is specific for an infectious microbe expressing at least one of the epitopes of SEQ ID NO: 2, 3, 4, 7, 17 and 31 wherein the method comprises contacting a precursor CTL with an immunogenic peptide selected from a group comprising the peptide immunogens of claims 6 and 7 under conditions that generate a CTL response to the microbe.

13. A method for inducing a CTL response *in vitro* that is specific for an infectious microbe expressing at least one of the epitopes of SEQ ID NO: 2, 3, 4, 7, 17 and 31 wherein the method comprises contacting a precursor CTL with an antigen presenting cell that has exogenously acquired the immunogen of claim 8.

14. A method of inducing a CTL response *in vitro* that is specific for an infectious microbe expressing at least one of the epitopes of SEQ

ID NO: 2, 3, 4, 7, 17 and 31 wherein the method comprises contacting a precursor CTL with an antigen presenting cell that is expressing a polynucleotide of claim 10 or 11.

15 15. A method of treating a subject infected with an infectious microbe expressing at least one of the epitopes of SEQ ID NO: 2, 3, 4, 7, 17 and 31 wherein the CTLs induced by the methods of claims 12, 13, or 14 are administered in an amount sufficient to destroy the infectious microbes through direct lysis or to effect the destruction of the infectious microbes indirectly through the elaboration of cytokines.

10 16. The method of claims 12, 13, 14 and 15 wherein said infectious microbe is selected from the group consisting of *Mycobacteria*, *Rickettsia*, *Chlamydia*, *Trypanosoma*, *Helicobacter*, *Leishmania*, *Trichomonas*, *Synechoccus*, and *Cowdria*.

15 17. The method of claim 16 wherein said infectious microbe is selected from the group consisting of *Mycobacterium tuberculosis*, *Trichomonas vaginalis*, *Synechoccus vulcanis*, and *Cowdria ruminatum*.

20 18. The method of claims 12, 13, 14 and 15 wherein said infectious microbe is selected from the group consisting of *M. leprae*, *M. paratuberculosis*, *Brucella abortus*, *Leptospira interrogans*, *Legionella pneumophila*, *Coxiella burnetii*, *Staphylococcus aureus*, *Salmonella typhi*, *Yersinia enterocolitica*, *Neisseria meningitidis*, *Neisseria gonorrhoeae*, *Haemophilus influenzae* and *Pseudomonas aeruginosa*.

25 19. The method of claims 12, 13, 14 and 15 wherein said infectious microbe is a microbe selected from the group of organisms of Table 4.

20. The method of claim 19 wherein said bacterium is a bacterium.

21. The method of claims 20 wherein said bacterium is a member of the genus *Mycobacterium*.

22. A method for inducing a CTL response in a subject comprising administering an immunogen comprising a peptide having an amino acid sequence selected from the group consisting of (a) SEQ ID NO: 2, 3, 7, 17 and 31; (b) a sequence differing from (a) by at least one but no more than 2 amino acids; (c) SEQ ID NO: 4; (d) a sequence differing from (c) by at least one but no more than 2 amino acids at least one CTL epitope, and wherein said immunogen is other than a bacterial heat shock protein 65 and wherein said peptide is other than a peptide contained in human heat shock protein 65.

23. A method for inducing a CTL response in a subject, comprising administering to a patient suffering from a microbial infection or at risk of such infection a polynucleotide encoding an immunogen recited in claim 22, including active fragments thereof, in an amount sufficient to induce a CTL response to infectious microbes.

24. The method of claim 24 wherein said polynucleotide is contained in a plasmid.

25. A method for inducing a CTL response in a subject, comprising administering to a patient suffering from a microbial infection or at risk of such infection a polynucleotide encoding a minigene an immunogen recited in claim 22 in an amount sufficient to induce a CTL response to infectious microbes.

Fig. 1

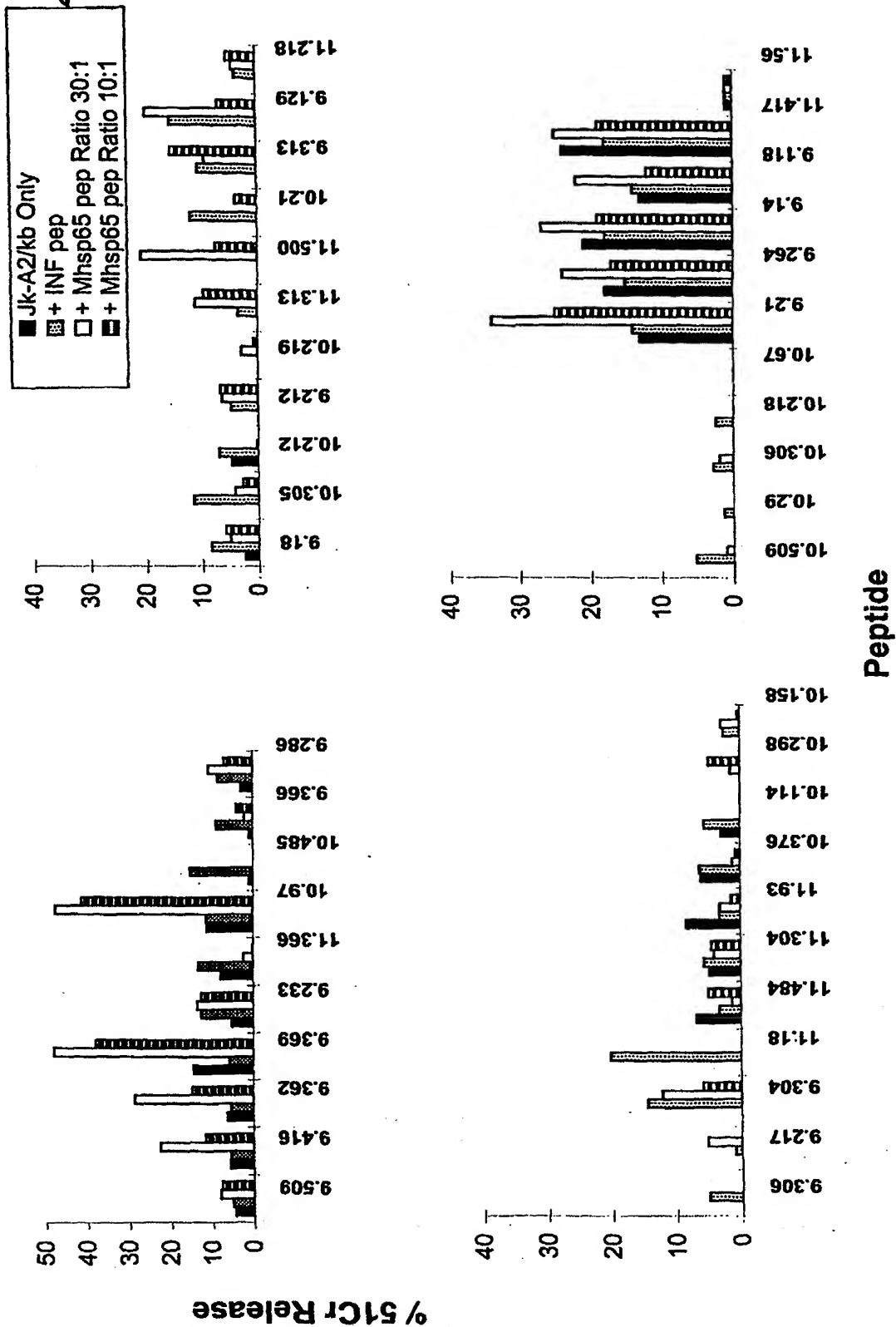
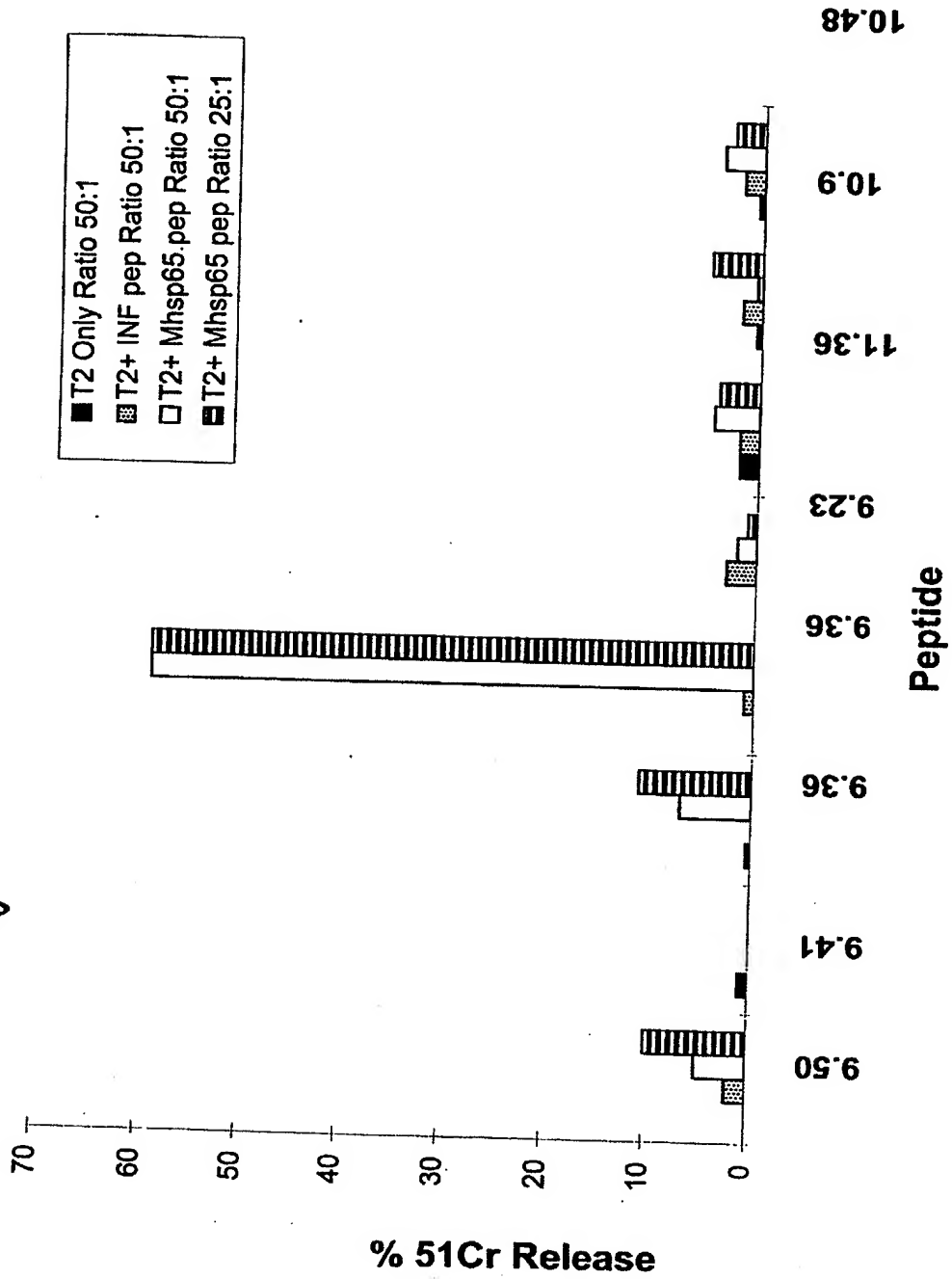


Fig. 2



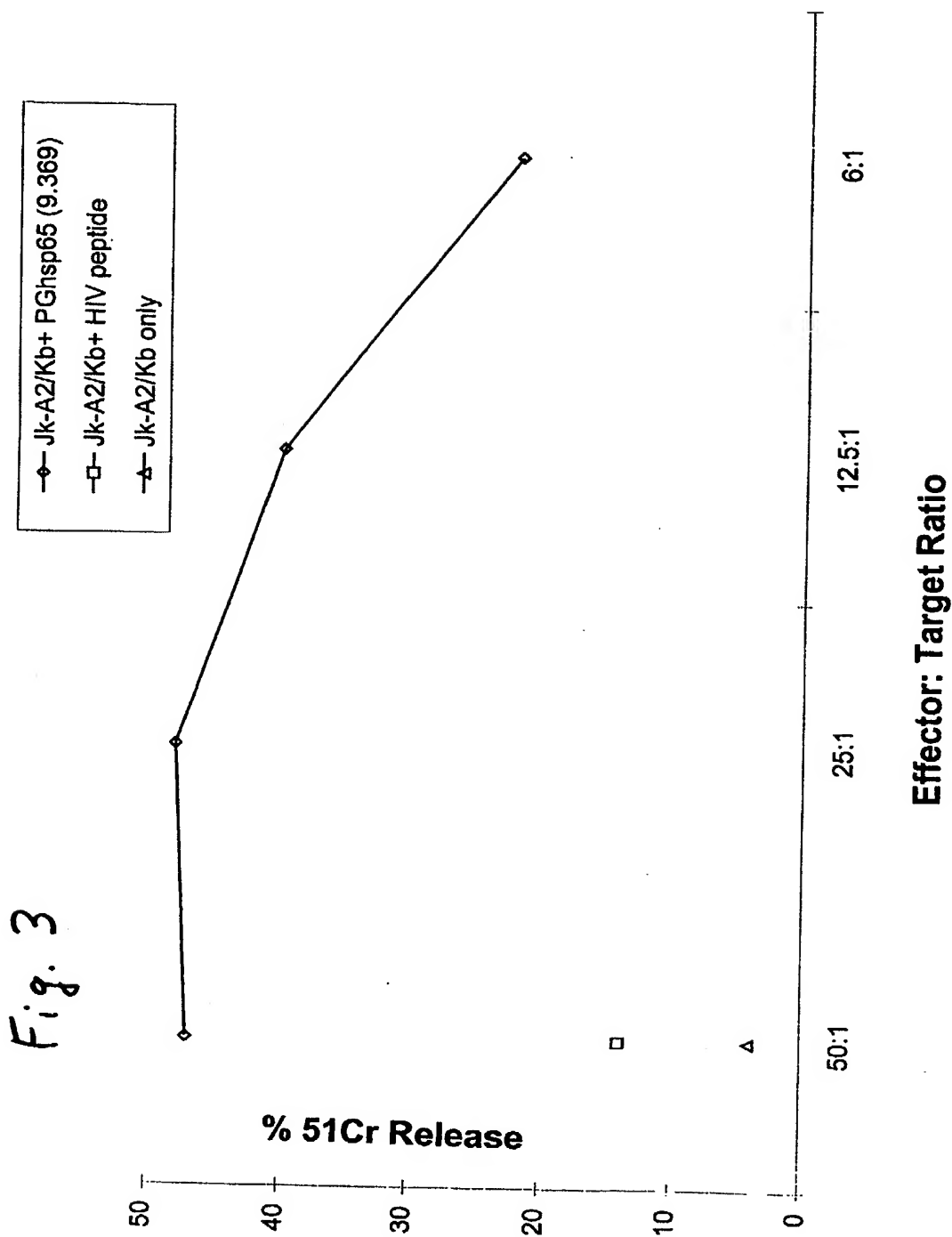


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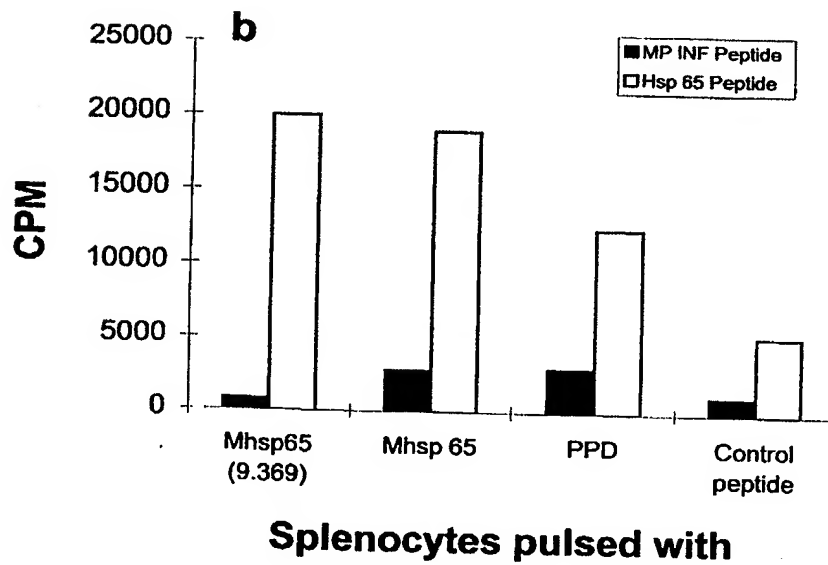
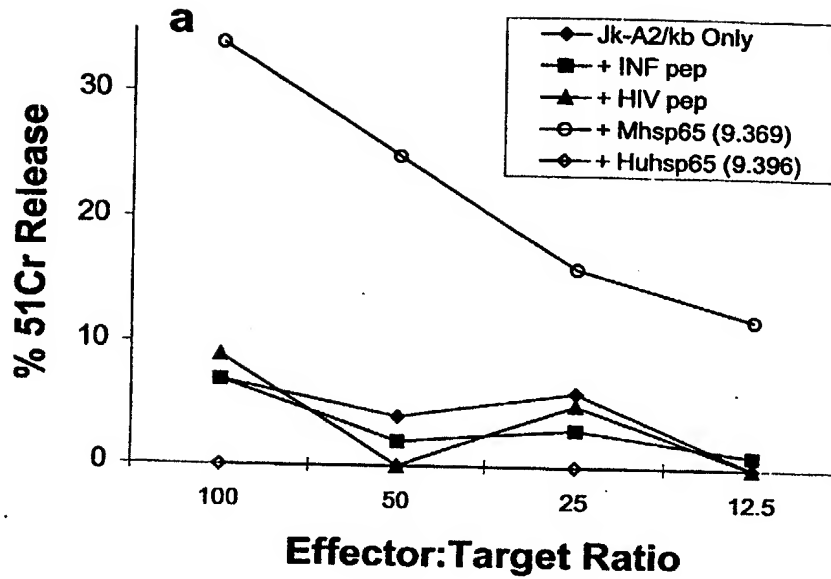


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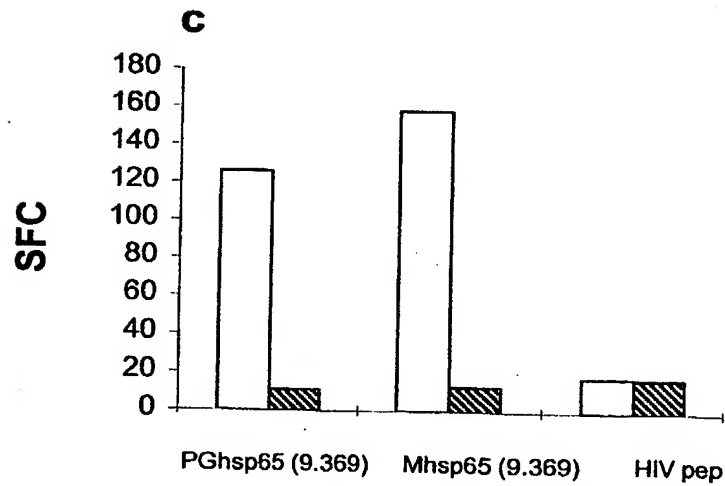
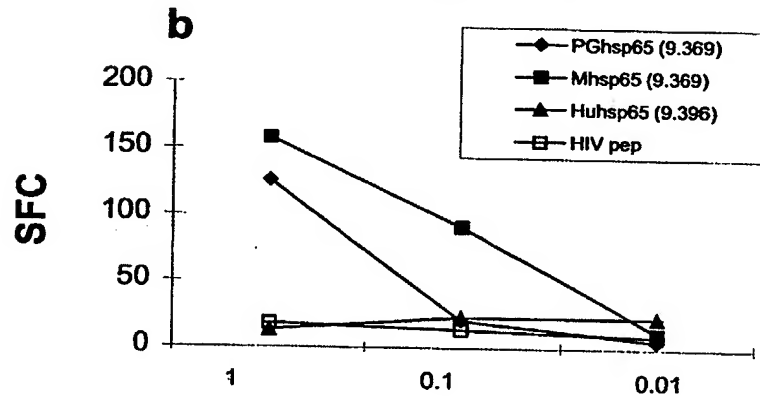
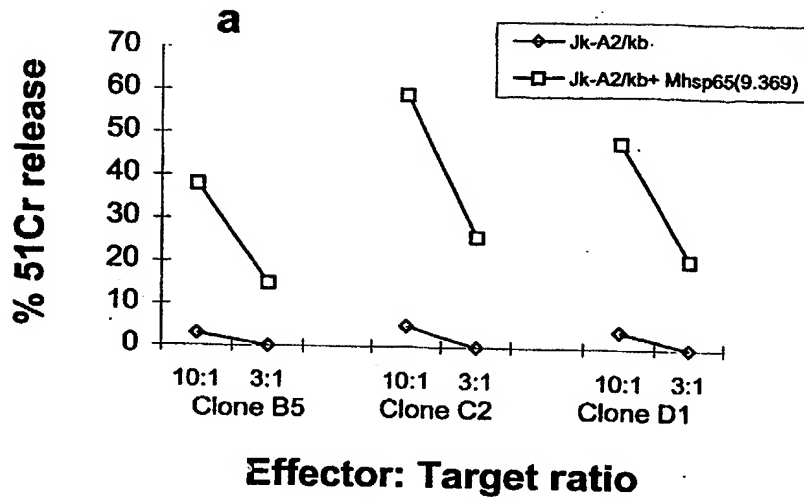


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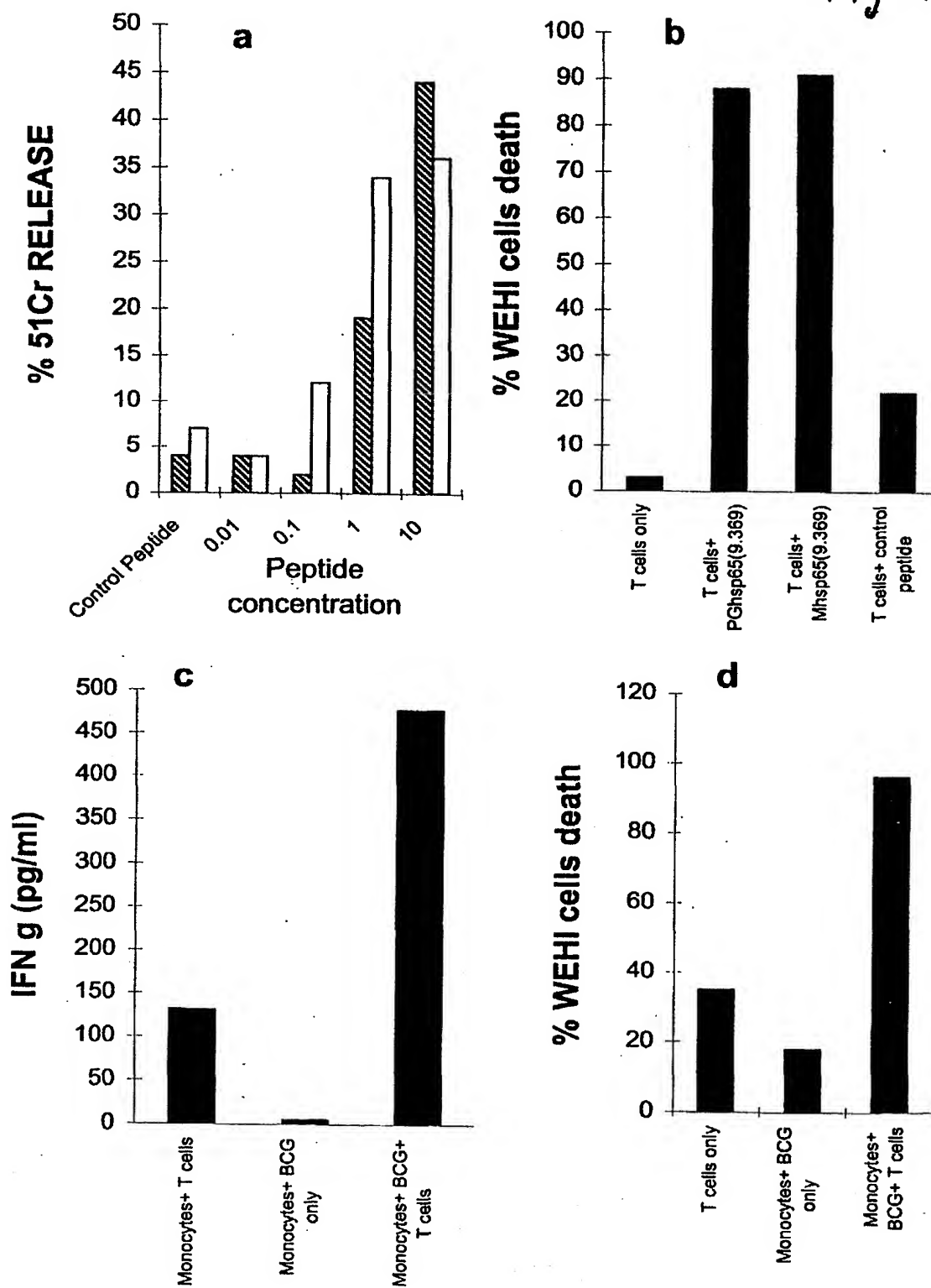
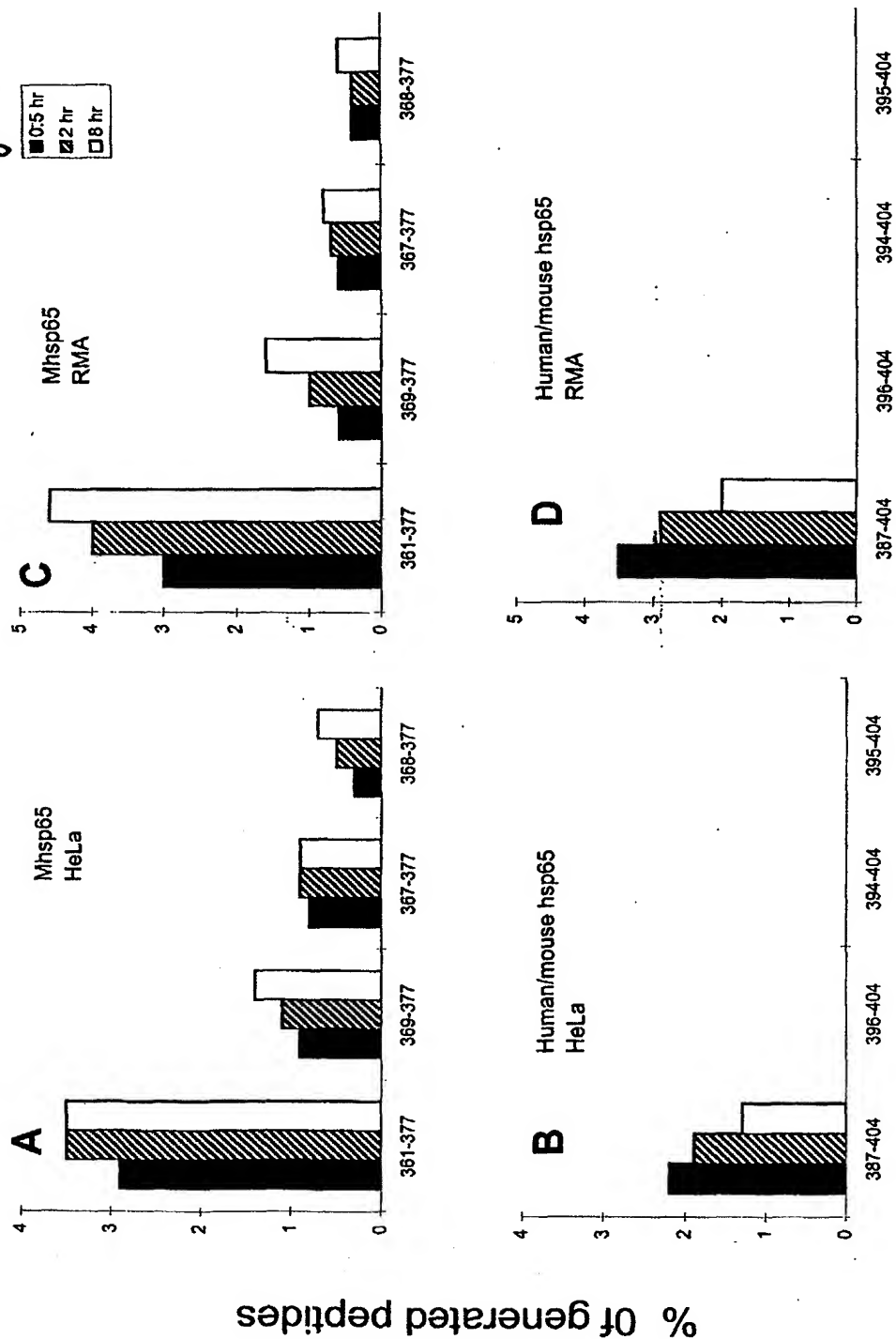


Fig. 7



SEQUENCE LISTING

<110> Kiessling, Rolf
Charo, Jehad M.
Otenhoff, Tom H.
Geluk, Annemiek

<120> Induction of Cytotoxic T Lymphocyte Response by HLA
Class IA Restricted Epitopes of Mycobacterial Heat
Shock Protein 65

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1 5 10

<210> 52
<211> 9
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Heat shock protein 65 epitopic peptide sequence.

<400> 52
Pro Leu Leu Ile Ile Ala Glu Asp Val
1 5

<210> 53
<211> 9
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Heat shock protein 65 epitopic peptide sequence.

<400> 53
Thr Leu Gly Pro Lys Gly Arg Asn Val
1 5

<210> 54
<211> 9

<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Heat shock protein 65 epitopic peptide sequence.

<400> 54
Glu Leu Glu Asp Pro Tyr Glu Lys Ile
1 5

<210> 55
<211> 9
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Heat shock protein 65 epitopic peptide sequence.

<400> 55
Lys Leu Ser Asp Gly Val Ala Val Leu
1 5

<210> 56
<211> 9
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Heat shock protein 65 epitopic peptide sequence.

<400> 56
Lys Leu Ala Gly Gly Val Ala Val Leu
1 5

<210> 57
<211> 10
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Heat shock protein 65 epitopic peptide sequence.

<400> 57
Phe Leu Pro Ser Asp Cys Phe Pro Ser Val
1 5 10

<210> 58
<211> 24
<212> PRT
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:Proteosome
digested heat shock peptide epitopes with
flankingamino acids.

<400> 58

Glu Lys Leu Gln Glu Arg Leu Ala Lys Leu Ala Gly Gly Val Ala Val
1 5 10 15

Ile Lys Ala Gly Ala Ala Thr Glu
20

<210> 59

<211> 21

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:Proteosome
digested heat shock peptide epitopes with
flankingamino acids.

<400> 59

Glu Lys Leu Gln Glu Arg Leu Ala Lys Leu Ala Gly Gly Val Ala Val
1 5 10 15

Ile Lys Ala Gly Ala
20

<210> 60

<211> 19

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:Proteosome
digested heat shock peptide epitopes with
flankingamino acids.

<400> 60

Glu Lys Leu Gln Glu Arg Leu Ala Lys Leu Ala Gly Gly Val Ala Val
1 5 10 15

Ile Lys Ala

<210> 61

<211> 18

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:Proteosome
digested heat shock peptide epitopes with
flankingamino acids.

<400> 61

Glu Lys Leu Gln Glu Arg Leu Ala Lys Leu Ala Gly Gly Val Ala Val
1 5 10 15

Ile Lys

<210> 62

<211> 17

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:Proteosome
digested heat shock peptide epitopes with
flankingamino acids.

<400> 62

Glu Lys Leu Gln Glu Arg Leu Ala Lys Leu Ala Gly Gly Val Ala Val
1 5 10 15

Ile

<210> 63

<211> 11

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:Proteosome
digested heat shock peptide epitopes with
flankingamino acids.

<400> 63

Glu Lys Leu Gln Glu Arg Leu Ala Lys Leu Ala
1 5 10

<210> 64

<211> 10

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:Proteosome
digested heat shock peptide epitopes with
flankingamino acids.

<400> 64

Glu Lys Leu Gln Glu Arg Leu Ala Lys Leu
1 5 10

<210> 65

<211> 19

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:Proteosome
digested heat shock peptide epitopes with
flankingamino acids.

<400> 65

Ala Lys Leu Ala Gly Gly Val Ala Val Ile Lys Ala Gly Ala Ala Thr
1 5 10 15

Glu Val Glu

<210> 66

<211> 18

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:Proteosome
digested heat shock peptide epitopes with
flankingamino acids.

<400> 66

Lys Leu Ala Gly Gly Val Ala Val Ile Lys Ala Gly Ala Ala Thr Glu
1 5 10 15

Val Glu

<210> 67

<211> 16

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:Proteosome
digested heat shock peptide epitopes with
flankingamino acids.

<400> 67

Ala Gly Gly Val Ala Val Ile Lys Ala Gly Ala Ala Thr Glu Val Glu
1 5 10 15

<210> 68

<211> 15

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:Proteosome
digested heat shock peptide epitopes with
flankingamino acids.

<400> 68

Gly Gly Val Ala Val Ile Lys Ala Gly Ala Ala Thr Glu Val Glu
 1 5 10 15

<210> 69
 <211> 23
 <212> PRT
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence:Proteosome
 digested heat shock peptide epitopes with
 flankingamino acids.

<400> 69
 Lys Leu Gln Glu Arg Leu Ala Lys Leu Ala Gly Gly Val Ala Val Ile
 1 5 10 15

Lys Ala Gly Ala Ala Thr Glu
 20

<210> 70
 <211> 13
 <212> PRT
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence:Proteosome
 digested heat shock peptide epitopes with
 flankingamino acids.

<400> 70
 Leu Ala Lys Leu Ala Gly Gly Val Ala Val Ile Lys Ala
 1 5 10

<210> 71
 <211> 11
 <212> PRT
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence:Proteosome
 digested heat shock peptide epitopes with
 flankingamino acids.

<400> 71
 Ala Lys Leu Ala Gly Gly Val Ala Val Ile Lys
 1 5 10

<210> 72
 <211> 11
 <212> PRT
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence:Proteosome

digested heat shock peptide epitopes with
flankingamino acids.

<400> 72

Leu Ala Lys Leu Ala Gly Gly Val Ala Val Ile
1 5 10

<210> 73

<211> 10

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:Proteosome
digested heat shock peptide epitopes with
flankingamino acids.

<400> 73

Ala Lys Leu Ala Gly Gly Val Ala Val Ile
1 5 10

<210> 74

<211> 20

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:Proteosome
digested heat shock peptide epitopes with
flankingamino acids.

<400> 74

Lys Glu Lys Leu Asn Glu Arg Leu Ala Lys Leu Ser Asp Gly Val Ala
1 5 10 15

Val Leu Lys Val
20

<210> 75

<211> 17

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:Proteosome
digested heat shock peptide epitopes with
flankingamino acids.

<400> 75

Glu Lys Leu Asn Glu Arg Leu Ala Lys Leu Ser Asp Gly Val Ala Val
1 5 10 15

Leu

<210> 76
<211> 17
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Proteosome
digested heat shock peptide epitopes with
flankingamino acids.

<400> 76
Lys Glu Lys Leu Asn Glu Arg Leu Ala Lys Leu Ser Asp Gly Val Ala
1 5 10 15

Val

<210> 77
<211> 13
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Proteosome
digested heat shock peptide epitopes with
flankingamino acids.

<400> 77
Lys Glu Lys Leu Asn Glu Arg Leu Ala Lys Leu Ser Asp
1 5 10

<210> 78
<211> 11
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Proteosome
digested heat shock peptide epitopes with
flankingamino acids.

<400> 78
Lys Glu Lys Leu Asn Glu Arg Leu Ala Lys Leu
1 5 10

<210> 79
<211> 18
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Proteosome
digested heat shock peptide epitopes with
flankingamino acids.

<400> 79

Ala Lys Leu Ser Asp Gly Val Ala Val Leu Lys Val Gly Gly Thr Ser
 1 5 10 15

Asp Val

<210> 80

<211> 15

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:Proteosome
 digested heat shock peptide epitopes with
 flankingamino acids.

<400> 80

Ser Asp Gly Val Ala Val Leu Lys Val Gly Gly Thr Ser Asp Val
 1 5 10 15

<210> 81

<211> 13

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:Proteosome
 digested heat shock peptide epitopes with
 flankingamino acids.

<400> 81

Gly Val Ala Val Leu Lys Val Gly Gly Thr Ser Asp Val
 1 5 10

<210> 82

<211> 9

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:Proteosome
 digested heat shock peptide epitopes with
 flankingamino acids.

<400> 82

Ser Asp Gly Val Ala Val Leu Lys Val
 1 5

<210> 83

<211> 26

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:Proteosome

digested heat shock peptide epitopes with
flanking amino acids.

<400> 83

Gln Asn Ala Ala Ser Ile Ala Gly Leu Phe Leu Thr Thr Glu Ala Val
1 5 10 15

Val Ala Asp Lys Pro Glu Lys Glu Lys Ala
20 25

<210> 84

<211> 26

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: 26-mer peptide
from Mhsp65 protein.

<400> 84

Glu Lys Leu Gln Glu Arg Leu Ala Lys Leu Ala Gly Gly Val Ala Val
1 5 10 15

Ile Lys Ala Gly Ala Ala Thr Glu Val Glu
20 25

<210> 85

<211> 26

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: 26-mer peptide
from Huhsp65 protein.

<400> 85

Lys Glu Lys Leu Asn Glu Arg Leu Ala Lys Leu Ser Asp Gly Val Ala
1 5 10 15

Val Leu Lys Val Gly Gly Thr Ser Asp Val
20 25